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Molecular and functional studies on human embryo implantation – Targets for infertility and fertility regulation

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MOLECULAR AND FUNCTIONAL STUDIES ON HUMAN EMBRYO IMPLANTATION – TARGETS FOR INFERTILITY AND FERTILITY REGULATION

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"It is better to live your own destiny imperfectly than to live an imitation of somebody's life with perfection"

The Bhagavad Gita

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Dear Mom and Dad, I have achieved what you dreamt for many years, by the time I reached your goals you left this world forever and I am missing you so badly each and every second in my life. But Mom and Dad, I am so much of what I learned from you. You'll be with me like a handprint on my heart forever. I have no words to acknowledge the sacrifices you made and the dreams you had to let go, just to give me a shot at achieving mine. My love towards you will be beyond stars, beyond the space, beyond the depth of the ocean, beyond the speed of light, beyond the heat of the sun. I am dedicating this thesis to you.

To my beloved parents❤

ABSTRACT

Background

An estimated one in four couples globally suffers from infertility or fertility related issues and rate of global infertility is about 10-15%. Despite the best optimization of assisted reproductive technique the pregnancy rate is not more than 30%. Poor understanding of the complex molecular interactions between the blastocyst and the receptive endometrium is one of the major reasons for unexplained infertility. Understanding the molecular mechanisms of human embryo implantation helps in improving pregnancy rates, management of infertility issues and helps in regulation of fertility by novel methods.

Aim

The overall aim of this thesis is to expand the understanding of various factors that affects endometrial receptivity and human embryo implantation process. The specific aims of the thesis are to explore the role of leukemia inhibitory factor (LIF) in implantation and viability of the human embryo and, to study the actions of ulipristal acetate (UPA) and two low doses of mifepristone on endometrial receptivity and human embryo implantation using an *in vitro* three-dimensional (3D) endometrial co-culture model, in addition to study progesterone regulated transcriptomic signature in epithelial and stromal compartments.

Materials, methods and results

Study 1 is an *in vitro* exploratory study of the role of LIF in human embryo implantation and its viability by using potent LIF inhibitor, polyethylene glycated leukemia inhibitory factor antagonist (PEGLA) in a 3D endometrial cell co-culture model. Inhibition of LIF by PEGLA significantly reduced blastocyst attachment to endometrial constructs and triggered apoptosis of blastocysts by down regulating embryonic AKT and up regulating caspase 3 as analyzed by immunofluorescence and RTPCR. Studies 2 and 3 were exploratory studies on endometrial receptivity and human embryo implantation process after treatment with 200 ng/ml UPA, a dose used for emergency contraception (study 2) and two low doses of mifepristone (0.5µM and 0.05µM, study 3) using an *in vitro* 3D endometrial cell co-culture model. Selected endometrial receptivity markers were analyzed by RTPCR from the endometrial constructs. The main findings of study 2 were that there was no significant difference in the blastocyst attachment rate to endometrial constructs when compared between UPA treated group (5/10 blastocysts attached) and control group (7/10 blastocysts attached). Of the studied 17 endometrial receptivity markers, HBEGF and IL6 were significantly upregulated and HAND2, OPN, CALCR and FGF2 were down- regulated with UPA treatment. The main findings of study 3 were that none of the embryos in 0.5µM of mifepristone attached to the endometrial constructs, whereas 4 out of 10 in 0.05 µM group and 7 out of 10 embryos in the control group attached to the cultures. Most of the studied receptivity markers were significantly altered with mifepristone exposure in a similar direction in both the treatment groups. Study 4 explored large-scale progesterone regulated

transcriptomic signature in epithelial and stromal compartments by laser capture microdissection and microarray analysis in receptive and non-receptive (treatment with 200 mg mifepristone on LH+2) endometrium. Expression of Metallothioneins (MT1G and MT2A) and Ectonucleotide pyrophosphatase/phosphodiesterase 3 (ENPP3) was significantly downregulated in both stromal cells and glands, whereas SFRP4 was upregulated. ENPP3 protein and mRNA expression was significantly down regulated in epithelial compartment of non-receptive endometrium, but no stromal immunostaining was detected in either receptive or non-receptive endometrium. ENPP3 protein was observed in glycosylated form in both the endometrial tissue lysates and uterine fluid. The expression pattern of ENPP3 was similar to progesterone secretion - high in mid-secretory and low in proliferative phase. *In vitro* functional assay using 3D cell cultures confirmed the receptivity of the endometrial construct falling in line with the expression of ENPP3.

Conclusion

LIF plays a critical role in the process of human embryo implantation and viability of the blastocyst. UPA at a dose used as emergency contraception (30 mg single dose) does not affect endometrial receptivity and embryo implantation. Mifepristone at a concentration of 0.5μM affected endometrial receptivity and inhibited embryo implantation whereas 0.05μM mifepristone affected the studied genes known to be involved in endometrial receptivity, but had no effect on embryo implantation. ENPP3 is proposed as a novel molecular marker of progesterone regulated endometrial receptivity.

LIST OF SCIENTIFIC PAPERS

I. **Polyethylene glycated leukemia inhibitory factor antagonist inhibits human blastocyst implantation and triggers apoptosis by down-regulating embryonic AKT**
Lalitkumar S, **Boggavarapu NR**, Menezes J, Dimitriadis E, Zhang JG, Nicola NA, Gemzell-Danielsson K, Lalitkumar LP
Fertil Steril. 2013 Oct;100(4):1160-9. doi: 10.1016/j.fertnstert.2013.06.023. Epub 2013 Jul 19.

II. **Effects of ulipristal acetate on human embryo attachment and endometrial cell gene expression in an *in vitro* co-culture system.**
Berger C*, **Boggavarapu NR***, Menezes J, Lalitkumar PG, Gemzell-Danielsson K
Hum Reprod. 2015 Apr;30(4):800-11. doi: 10.1093/humrep/dev030. Epub 2015 Mar 3.

III. **Effects of low doses of mifepristone on human embryo implantation process in a three-dimensional human endometrial *in vitro* co-culture system.**
Boggavarapu NR*, Berger C*, von Grothusen C, Menezes J, Gemzell-Danielsson K, Lalitkumar PG.
Contraception. 2016 Aug;94(2):143-51. doi: 10.1016/j.contraception. 2016.03.009. Epub 2016 Mar 18.

IV. **Compartmentalized gene expression profiling of receptive endometrium reveals progesterone regulated ENPP3 is differentially expressed and secreted in glycosylated form.**
Boggavarapu NR, Lalitkumar S, Joshua V, Kasvandik S, Salumets A, Lalitkumar PG, Gemzell-Danielsson K.
Sci Rep. 2016 Sep 26;6:33811. doi: 10.1038/srep33811.

* *joint first authorship*

CONTENTS

LIST OF ABBREVIATIONS

ABCG2 - ATP binding cassette subfamily G member 2

ART – Assisted reproductive technology

BMP2 - Bone morphogenetic protein 2

CaM - Calmodulin

cAMP – cyclic AMP

CAMs – Cell adhesion molecules

CLDN4 - Claudin 4

COUP-TF2 - Chicken ovalbumin upstream promoter transcription factor 2

COX - Cyclooxygenases

CRH - Corticotropin releasing hormone

CSF – Colony stimulating factor

EC - Emergency contraception / Emergency contraceptive

ECM – Extracellular matrix

EGFR - Epidermal growth factor receptor

EMMPRIN - Extracellular matrix metalloproteinase inducer

ENaC – Epithelial sodium channels

ENPP3 - Ectonucleotide pyrophosphatase/phosphodiesterase 3

ER – Estrogen receptor

ER Map - Endometrial receptivity map

ERA - Endometrial receptivity array

ET – Embryo transfer

FABP4 - Fatty acid binding protein 4

FGF – Fibroblast growth factor

FOXO1 - Forkhead box protein O1

FSH - Follicular stimulating hormone

FUT - Fucosyl transferases

G-CSF - Granulocyte-colony stimulating factor

Gal - Galectins

GC-MS - gas chromatography- mass spectrometry

GM-CSF - Granulocyte-macrophage colony stimulating factor

GnRH – Gonadotropin releasing hormone

GPCR - G-protein coupled receptor

HAND2 - Heart and neural crest derivatives-expressed protein 2

HB-EGF - Heparin binding-epidermal growth factor

hCG – human chorionic gonadotropin

Hox - Homeobox genes

HPA - Hypothalamo-pituitary axis

HPLC - High-pressure / performance liquid chromatography

ICAM-1 - Intercellular adhesion molecule-1

IFN-γ - Interferon-γ

IGF - Insulin like growth factor

IGFBP1 - Insulin like growth factor binding protein 1

IgSF - Immunoglobulin superfamily

IHC – Immunohistochemistry

IL – Interleukin

IRE - Insulin response element

IVF - *In vitro* fertilization

JAK/STAT - Janus Kinase/Signal transducer and activator of transcription

KGF – Keratinocyte growth factor

KRT - Keratin

LC-MS/MS - Liquid chromatography-tandem mass spectrometry

LCMD - Laser capture micro dissection

LH – Luteinizing hormone

LIF - Leukemia inhibitory factor

LIFR - Leukemia inhibitory factor receptor

LNG - Levonorgestrel

LPA - Lysophosphatidic acid

M-CSF - Macrophage - colony stimulating factor

MAO – Mono amino oxidase

MAPK - Mitogen activated protein kinase

miR - microRNA

MMP – Matrix metalloproteinases

MRP1 - Motility related protein 1

MSC – Mesenchymal stem/stromal cells

MUC - Mucin

NGS – Next generation sequencing

NIR - Near infrared spectroscopy

NMR - Nuclear magnetic resonance

OPN - Osteopontin

PCOS - Polycystic ovary syndrome

PDGF – Platelet derived growth factor

PEGLA - Polyethylene glycated leukemia inhibitory factor antagonist

PGE2 - Prostaglandin E2

PGs – Prostaglandins

PP14 - Placental protein 14

PR – Progesterone receptor

PRL - Prolactin

PRM - Progesterone receptor modulator

RIF – Recurrent implantation failure

SGK1 - Serum/Glucocorticoid regulated kinase 1

sICAM-1 - soluble ICAM-1

SIRT1 - Sirtuin 1

SP – Side population cells

SPRM - Selective progesterone receptor modulators

SSEA-1 - Stage–specific embryonic antigen-1

SUSD2 - Sushi domain containing 2

TGF-β - Transforming growth factor-β

TIMP - Tissue inhibitors of metalloproteinases

tTG - Tissue transglutaminase

uNK – uterine Natural Killer cells

UPA - Ulipristal acetate

VCAM-1 - vascular cell adhesion molecule-1

VEGF – Vascular endothelial growth factor

VEGFR - VEGF receptor

WHO – World health organization

WOI – Window of implantation

I. Introduction

Fertility is the natural ability to produce offspring. World Health Organization (WHO) defines infertility as "a disease of reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (six months if the woman is over age 35) 'or' inability to carry a pregnancy to a live birth". Infertility is classified as primary infertility – a woman who never conceived, and secondary infertility – a failure to conceive after previous pregnancy. Each of the following factors contributes equally (about 1/3rd) to infertility - female partners, male partners, or the combination of both. Many different causes of infertility include age, genetic abnormalities, hormonal abnormalities, lack of regular ovulation, anatomical defects like blocked fallopian tubes, problems with uterine cavity, cervical defects, peritoneal factors, endometriosis, uterine fibroids, polycystic ovary syndrome or unexplained (1). An estimated one in four couples, numbering 48.5 million couples globally suffered from infertility or subfertility related issues in the year 2010 alone (2).

Research in reproductive sciences has made the management of fertility or sub fertility easier in the recent years. As we celebrate the Noble Prize 2010 (RG Edwards, 2010) technique of Assisted Reproductive Technology (ART) which has added a new dimension in treating childless couples, we should notice that the rate of global infertility is still about 10-15% (Reproductive Health Outlook, 2005). Despite the best optimization of critical events of ART, pregnancy rate is not more than 30% (3). As the issue of infertility causes great emotional and economic consequences in the economically developed world, the issue of fertility regulation is a major burden in the economically developing countries. One of the reasons for the unexplained infertility is poor understanding of the synchronized, complex molecular interactions taking place between the pre-implantation embryo and the receptive endometrium during the window of implantation (WOI).

1.1 Uterus

Uterus is divided into an upper part- the body and a lower part – the cervix that is continuous with upper vagina. It is composed of three layers – perimetrium which is the outside layer of serosa, myometrium, the middle muscular layer and endometrium, the inner layer. Endometrium is a complex and dynamic tissue that undergoes regeneration, differentiation, and shedding in each menstrual cycle during the reproductive life of a woman.

1.1.1 Endometrium

Histologically endometrium is divided into the bottom one-third permanent stratum basalis and the superficial two-thirds stratum functionalis. The upper functionalis layer is highly sensitive to the fluctuating ovarian hormones, estrogen and progesterone, and it is this layer that degenerates and shed during the menstrual bleeding (if there is no implantation) during each menstrual cycle. Post menstruation, functionalis layer is regenerated from basalis layer. Endometrium is composed of mostly stromal & epithelial cells (luminal and glandular) and to a lesser extent, stem cells, endothelial cells and immune cells - macrophages and uterine natural killer cells (uNK cells).

1.1.1.1 Epithelial compartment

The endometrial epithelial cells play a key role in embryo implantation by selectively allowing the embryo implantation during WOI. Outside WOI these cells are refractory to embryo implantation due to the presence of hormonally controlled protective glycocalyx molecules (4). Epithelial compartment is classified into the superficial luminal epithelial layer and glandular epithelium within the stromal compartment. Luminal epithelium serves as a barrier against infections and as a surface for blastocyst attachment and implantation. Glandular epithelium is composed of a single layer of ciliated columnar epithelial cells that are phenotypically different. Morphology of glandular epithelium change constantly in accordance with the ovarian hormone secretions throughout various phases of menstrual cycle. During proliferative phase where estrogen is dominant, the glands appear straight and long, during secretory phase the glands become coiled and produce secretions under the influence of progesterone.

1.1.1.2 Stromal compartment

Stromal cells, fibroblastic in nature, are located in the connective tissue of the endometrium that is made up of proteoglycans and collagen. Thickness of stromal compartment varies under the ovarian hormonal influences. Estrogen increases the proliferation of stromal cells during the proliferative phase; progesterone in the secretory phase reduces stromal proliferation. Upon successful blastocyst implantation the stromal cells complete decidualization and terminally differentiate (5). Stromal cells are loosely packed in the functionalis layer to facilitate blastocyst implantation whereas densely packed in the basalis layer of the endometrium. Stromal cells secrete various tissue-remodeling substances that include matrix metalloproteinases (MMP-2 and MMP-4), tissue inhibitors of metalloproteinases (TIMPs), growth factors and cytokines. MMPs expression in the stroma is mainly regulated by a cell surface glycoprotein present in the luminal and glandular epithelium known as extracellular matrix metalloproteinase inducer (EMMPRIN)(6).

1.1.1.3 Immune cells

The predominant immune cells - macrophages/dendritic cells, T-Lymphocytes, neutrophils, mast cells and uNK cells are located in the stromal compartment. Sex steroid hormones influence immune system in female reproductive tract, though the leucocytes lack receptors for estrogen and progesterone. Interestingly, the immune cells provide immunity against vaginal pathogens in lower tract whereas immune tolerance towards the sperm and embryo is maintained in upper tract (7). Very few B-lymphocytes are seen in endometrium throughout the menstrual cycle in comparison to T lymphocytes.

Elevated levels of uNK cells is observed in late secretory phase and further increased during early pregnancy (8). A positive correlation is observed between the circulating progesterone levels and uNK cells and a reduced number of uNK cells are seen in endometrium with anti progestin Asoprisnil treatment. With antiprogestin treatment, IL-5 pathway is identified to be involved in the development, function and differentiation of immature uNK to mature uNK cells (9). Though uNK cells lack the progesterone receptors, it is suggested that progesterone acts indirectly through cytokines and soluble factors secreted by stromal cells (10). Macrophages and dendritic cells are seen throughout all the phases of menstrual cycle, significant number of macrophages is especially seen around the glands during menstrual phase and notably at the implantation site.

1.1.1.4 Endothelial cells

Sex hormones regulate endothelial cell population in the endometrial vasculature. Angiogenesis is formation of new blood vessels either growing from pre-existing vessels or by intussusception i.e. formation of new vessels by division of pre existing vessels (11). Angiogenesis plays a crucial role in menstrual cycle during endometrial maturation and regeneration after menstruation. Vascular endothelial growth factor (VEGF) is considered the most important stimulator of angiogenesis and has been shown to inhibit apoptosis in endothelial cells *in vitro*.

Endothelial cells in human endometrium express progesterone receptor (PR) and estrogen receptor-β (ERβ). However, estrogen also acts indirectly by enhancing the expression of VEGF and fibroblast growth factor -2 (FGF-2). The role of progesterone in angiogenesis is controversial, *in vitro* it has been reported to have stimulatory as well as an inhibitory effect on endothelial cell proliferation (12). It has been proposed that progesterone acts through paracrine factors/mechanisms secreted by stromal cells that are close to vessels and treatment with anti-progestin mifepristone is shown to reduce the tube formation in endothelial cells (13).

1.1.1.5 Endometrial stem cells

Based on the unique properties of endometrium like tissue repair, regeneration and destruction during menstruation, it is believed that there is a population of tissue stem cells that is responsible for the regeneration of the functionalis layer of endometrium from the basalis layer. Stem cells are clonogenic, self-renewable, possess potential for differentiation and are proliferative. The stem cell population in endometrium includes mesenchymal stem/stromal cells (MSCs), bone marrow derived stem cells and side population cells (SP cells). To date, no specific markers have been identified for isolating endometrial epithelial progenitor cells, however recently stage–specific embryonic antigen-1 (SSEA-1/CD15) (13) was proposed as a marker for epithelial stem cells. SSEA-1+ cells exhibit adult stem cell properties like increased telomerase activity, longer telomeres; however it is not yet known whether the SSEA-1+ cells have clonogenic or self-renewal properties in the epithelial compartment. The minimum criteria for a cell to be identified as MSC is that the cell has to coexpress CD146 and CD140b (PDGFRβ) along with a single perivascular marker, sushi domain containing 2 (SUSD2) (W5C5 antibody) (14-16).

SP cells vary during each phase of menstrual cycle being highest in proliferative phase and least in late secretory phase (17). SP cells characteristically have rich ABCG2 (ATP binding cassette subfamily G member 2) expression and are located in functionalis and basalis layers near the vascular walls (18). Interestingly, the concept of bone marrow derived stem cells arises from the experimental animals and studies tracing donor specific markers following bone marrow transplantation; yet the role of these cells in endometrial regeneration is unknown (19, 20). Of many pathways involved in regulation of stem cell systems in uterus, Wnt/β-catenin signaling pathway is considered as a key pathway (21). Detailed knowledge of this pathway would help in understanding other stem cell specific molecular mechanisms involved in normal physiology and pathology of uterus.

1.2 Menstrual cycle

Menstrual cycle comprises the cyclical changes that take place in ovary, uterus, vagina and mammary glands once every 28 days during the reproductive age of the woman. Menstrual cycle is further classified as ovarian cycle, endometrial/uterine cycle, vaginal cycle and cyclical changes that occur in mammary glands (Figure 1).

1.2.1 Ovarian cycle

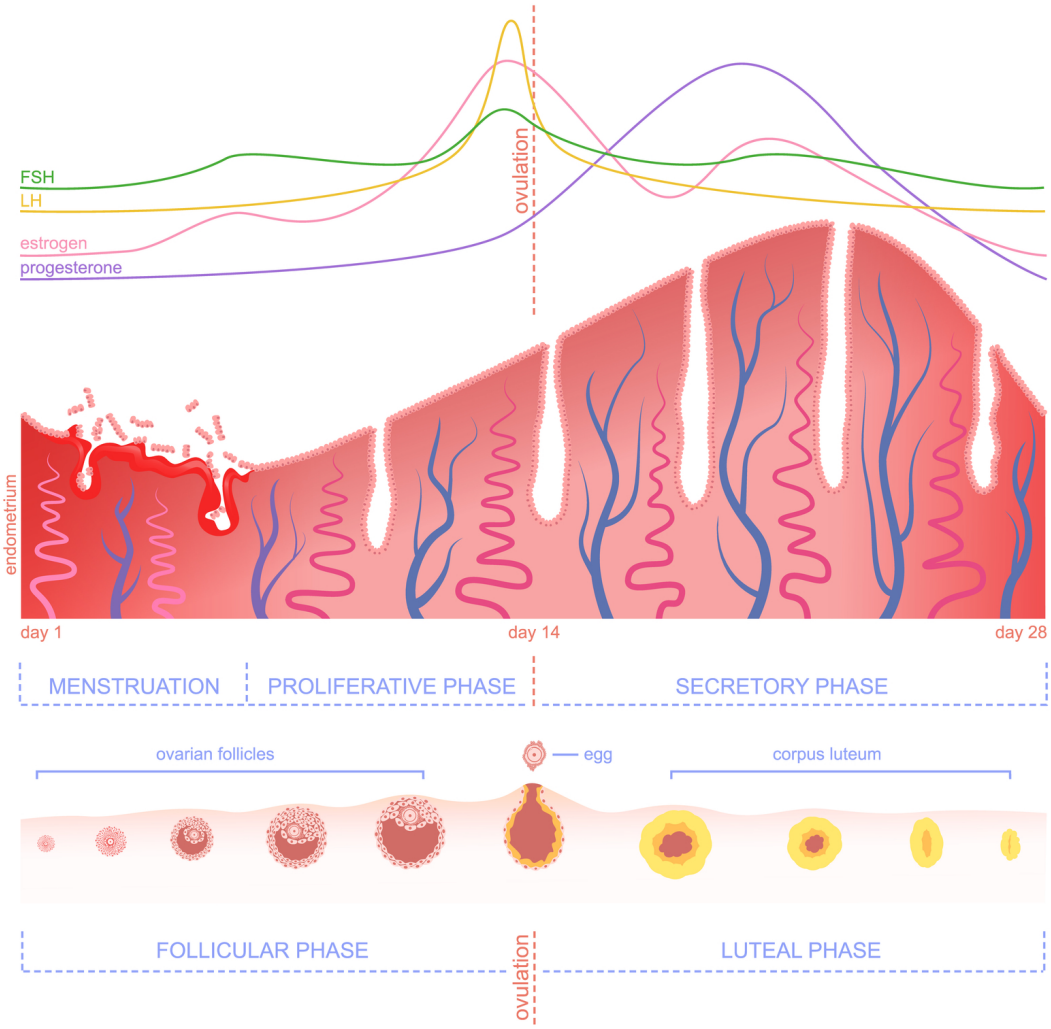
Ovarian cycle is the sequential changes that occur in the ovary during the menstrual cycle and is further classified into follicular phase and luteal phase with day of ovulation on the14^{th} day in a 28 days menstrual cycle.

During the follicular phase, under the influence of follicular stimulating hormone (FSH), 6-12 primary follicles start developing at the beginning of every cycle. Following early follicular phase, the primary follicles secrete follicular fluid rich in estrogens and the fluid cavity known as antrum appears. Pituitary FSH along with high estrogen concentration from the antral follicle promotes the appearance of LH receptors and stimulates the secretion of Luteinizing hormone (LH) in the granulosa cells of ovum. Subsequently estrogens, in combination with LH promote the growth of antral follicle and increase the size by 3-4 times. By the second week, one of the follicles becomes dominant follicle and all other follicles undergo atresia. The dominant follicle secretes more estrogen so that the plasma concentration of estrogen starts increasing, and the dominant follicle by the time of ovulation reaches a diameter of 1.5 -2 cm and is termed as mature follicle.

Increased estrogen concentration during the late follicular phase stimulates gonadotropin releasing hormone (GnRH) secretion and enhances the LH hormone secretion from the pituitary leading to a surge in the production of LH known as LH peak. The midcycle LH surge induces the ovulation, which occurs approximately on day 14, 10-12 hours post LH peak, and 24-36 hours after peak estrogen level.

In the luteal phase, LH surge stimulates the remaining granulosa cells and theca cells of the mature follicle to transform into lutein cells that are filled with lipid inclusions and appear yellow in color. This process is called ‘luteinization’ and the complete follicle is termed as corpus luteum. If fertilization fails to occur 7-8 days of ovulation, the corpus luteum starts involuting and looses its secretory activity becoming corpus albicans. As a result plasma concentrations of progesterone and estrogens start declining and the inhibitory effect of these ovarian hormones on GnRH secretion is released, starting a new cycle with rising FSH and LH hormones. On successful fertilization and implantation, the corpus luteum is maintained as corpus luteum of pregnancy that secretes high levels of progesterone and estrogen to maintain pregnancy.

Figure 1 : Menstrual cycle



1.2.2 Endometrial cycle

Endometrial cycle is the sequential morphological and molecular changes that take place in the endometrium during the menstrual cycle. Endometrial cycle is divided into proliferative and secretory phase separated by the day of ovulation. During the first five days of cycle, endometrium sheds off into the menstrual blood. After the menstrual phase, the endometrium is less than 2 mm thickness and under the influence of estrogens, proliferation of glands, blood vessels, stroma and luminal epithelium takes place, termed as proliferative phase, and the endometrium reaches a thickness of 4-5 mm by the day of ovulation.

During secretory phase or progestational phase, corpus luteum secretes much higher quantities of progesterone than estrogen. Estrogen during this phase causes slight additional cellular proliferation in endometrium and high progesterone concentration cause marked swelling and increase in the secretory activity of the cells. Under the influence of progesterone, glands increase in tortuosity and large quantity of secretory substances is deposited in the glands. The cytoplasm of stromal cells are packed with glycogen and lipids

and transform the structure, a process termed as decidualization. Increased blood supply ensues to endometrium with developing secretory activity. The purpose of all these observed changes of the secretory phase is to produce a secretory endometrium containing sizeable amounts of accumulated nutrients to provide optimal conditions for implantation of the embryo and for accommodating subsequent embryo development.

1.2.3 Decidualization

Decidualization is the morphological changes that are observed in stromal cells during the late luteal phase. The process starts around cycle day 23 and is independent of the presence or absence of blastocyst. If implantation occurs, decidualization plays an important role in formation of placenta by mediating the invasion of trophoblasts, and lack of decidualization lead to a failed placentation (22). Decidualization is characterized by transformation of elongated stromal cells or fibroblasts into larger and circular phenotype by the accumulation of glycogen and lipids, secreting numerous cellular products. Further changes that are seen during decidualization include presence of leukocytes and vascular changes in the maternal arteries. If no implantation occurs, the decidualized endometrial lining is shed in the menstruation.

In vitro, the stromal cells can be decidualized by progesterone treatment of estrogen primed cells, ligands of cyclic AMP (cAMP) pathway like Prostaglandin E2 (PGE2), LH, FSH and Relaxin hormone (23, 24). cAMP alone can induce decidualization if the stromal cells are obtained from the late luteal phase biopsy. In general prolactin (PRL), insulin like growth factor binding protein 1 (IGFBP1) and notch1 are considered as markers of decidualization.

1.3 Endometrial dating

Noyes endometrial histological dating (25) was considered a gold standard for endometrial dating. Hitchmann and Adler were the first to observe histological changes in the endometrium, which were further amplified by Schroeder, Novak, O'Leary and Bartelmez (25). Frankel and R.Meyer correlated these findings with the coincidental changes in ovary (25). Morphometric analysis was an improved version of histological dating proposed by Johannison in 1982 (26). It is an objective and quantitative technique that related the results to peripheral hormone levels. A total of 17 morphometric measurements are studied in this analysis and are compared with the chronological dating as defined by LH surge and shows a good correlation ($r = 0.98$). It is more accurate and reliable to date endometrium in comparison to criteria by Noyes et.al (27). To overcome the improbability of accuracy and utility with the histological dating other approaches such as whole genome molecular phenotyping (28), non invasive testing by high resolution endovaginal ultrasonography (29, 30) are employed and have been shown with good predictive value, better than histological dating.

1.4 Fertilization

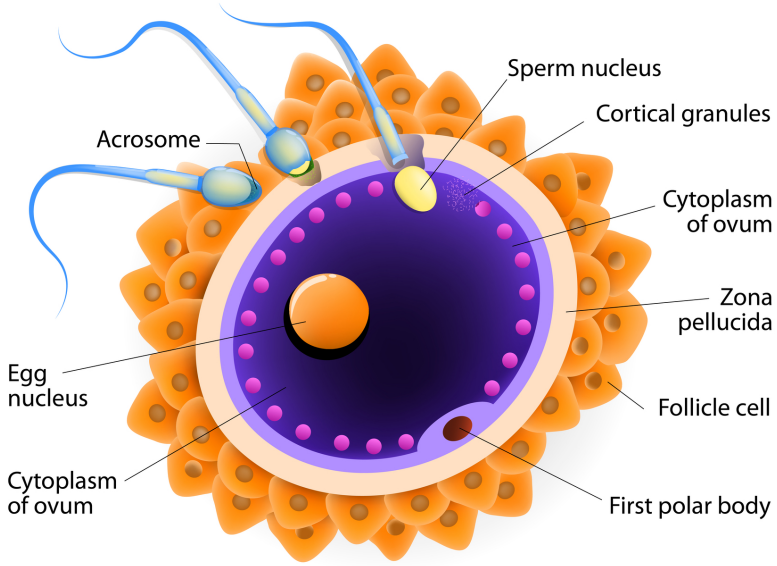
Fertilization is the process of the union of human ovum with sperm and this normally takes place in the ampulla of fallopian tube (Figure 2).

1.4.1 Mechanism of fertilization

Immediately after ovulation, around 14th day of menstrual cycle of a normal 28 day cycle, the ovum is released and carried into the ampulla of fallopian tube along with the cumulus oophorus. Ovary by secreting chemotactants attracts the sperms and GnRH that is produced locally by fallopian tube assists binding of sperm to the protective layer of ovum, the zona pellucida. The acrosomal cap of sperm secretes a trypsin like enzyme, acrosin, which disperses the corona of the ovum and permits the attachment of sperm to zona pellucida.

Penetration of zona pellucida by the successful sperm creates a block to entry of other sperms in sequential steps such as uptake of Ca++ into ovum, plasma membrane depolarization and release of proteases and glycosidases from the secretory granules of ovum and finally alters the zona surface glycoprotein, ZP3 that rejects the additional sperms rather than attracting. Therefore it prevents complete entrance of partially penetrated sperms, thereby preventing polyploidy, a condition in which more than two sets of homologous chromosomes is seen.

Figure 2: Mechanism of fertilization



On successful fertilization, the ovum undergoes second meiotic division releasing the polar body, leaving the ovum in a haploid state i.e. with 23 chromosomes. Fusion of sperm releases the nuclear chromatin material that combines with the haploid nucleus of ovum giving rise to diploid individual or zygote i.e. 46 chromosomes.

1.4.2 *In vitro* fertilization (IVF) and embryo grading

Since the evolution of IVF, embryologists grade the embryo for embryo transfer (ET) during IVF treatment based on the morphology and cleavage rates. Because of limitations in morphologic grading, the embryo secretion and metabolic consumption of the embryo spent media may give a better picture about the quality of the embryos (31). A positive correlation is seen with the nutrient uptake from the culture media and high morphological grade of embryo; however its use is limited because of high cost and need for highly trained experts (32). In view of these limitations, a non-invasive, rapid and consistent method was tried based on the metabolomics of the embryo-spent medium by Raman spectroscopy (33). However, in most IVF clinics Gardner and Schoolcraft's classification is widely used for assessing the quality of blastocyst and blastocyst with a minimum grade of 3BB suggested for ET clinically (34).

In Gardner grading system, each blastocyst is assigned a quality score based on three criteria namely 1) Blastocyst development stage- based on expansion 2) Inner cell mass (ICM) score 3) Trophectoderm (TE) score.

Table 1: Gardner and Schoolcraft's Embryo grading system

Expansion grade	**Blastocyst development and stage status**
1	Blastocoel cavity less than half the volume of the embryo
2	Blastocoel cavity more than half the volume of the embryo
3	Full blastocyst, cavity completely filling the embryo
4	Expanded blastocyst, cavity larger than the embryo, with thinning of the shell
5	Hatching out of the shell
6	Hatched out of the shell
ICM Grade	**Inner cell mass quality**
A	Many cells, tightly packed
B	Several cells, loosely grouped
C	Very few cells
TE grade	**Trophectoderm quality**
A	Many cells, forming a cohesive layer
B	Few cells, forming a loose epithelium
C	Very few large cells

1.5. Endometrial receptivity

Endometrial receptivity is defined as the time during which the endometrium is favorable for embryo implantation. This occurs in luteal phase approximately from 6th to 10th day after LH surge (35), which is also termed as window of implantation (36). During this period endometrium undergoes various molecular and structural changes influenced by hormones, growth factors and various molecular mediators. Any defects during this period, either in the embryo or the endometrium, will lead to implantation failure. In light of both ethical and technical issues, it is very difficult to identify site and mechanisms of implantation *in vivo*. Therefore, majority knowledge about implantation and embryo development is obtained from animal models; however, the limitation is that the reproductive physiology is different in different species, hence it is difficult to generalize these models to the process in humans.

The availability of biomarkers of endometrial receptivity is increasing exponentially owing to recent studies and advances that have opened avenues for the discovery of new biomarkers; however, a single unequivocal marker has not yet been established.

1.5.1 Biomarkers of endometrial receptivity and implantation

1.5.1.1 HORMONES

1.5.1.1.1 Estrogen

Priming of the endometrium by estrogen results in endometrial proliferation and induction of PRs that allow progesterone subsequently to induce endometrial receptivity. For normal development of the endometrium, presence of estrogen is important, although not necessarily in a large quantity (37). A high concentration of serum estrogen increases the risk of pregnancy complications such as abnormal placentation (38). Estrogen acts through the estrogen receptors (ER), which exist in two isoforms (ERα and ERβ). Of the two isoforms, ERα is dominant; in the case of a lack of ERα, uterus becomes hypoplastic and shows no response to estrogen treatment (39). No such effects are seen in the absence of ERβ (40).

Estrogen binds to membrane-associated G-protein-coupled receptor (GPCR) and promotes the release of nascent pro-heparin binding-epidermal growth factor (HB-EGF) that binds to epidermal growth factor receptor (EGFR) and activates downstream mitogen-activated protein kinases (MAPK) (41), resulting in a crosstalk with growth factors or insulin like growth factor-1 (IGF-1) cascades, stimulating proliferation.

Calbindin-D28k, an intracellular calcium-binding protein, is regulated by estrogen and is involved in the regulation of endometrial receptivity by altering the concentration of intracellular calcium ions (42). Calbindin-D28k expression is high during the proliferative

phase and at the time of sexual maturity, estrogen inhibits its expression in the uterus, suggesting that calbindin-D28k plays an important role in endometrial receptivity (43). A similar intracellular calcium-binding protein, Calmodulin (CaM) play a significant role in the contraction of smooth muscles in myometrium and a pivotal role in the proliferation of a variety of cells. Estrogens in combination with chorionic gonadotropins also increase the expression of Notch1, a decidualization marker (44). Absence of Notch-1 leads to apoptosis of stromal fibroblasts cells, uterine sloughing, and a reduction in cell survival and differentiation, which lead to decidualization defects affecting the pregnancy (45).

1.5.1.1.2 Progesterone

Progesterone is the key hormone that drives endometrial receptivity and interruption of luteal phase progesterone using anti-progestins makes the endometrium non receptive. Role of progesterone in maintenance of endometrial receptivity and pregnancy is originated from the progesterone receptor knockout mice (46). Progesterone mediates its actions in the target organs by progesterone receptors (PR). Affinity of progesterone towards PR is less than the affinity of estrogen towards its estrogen receptors (47). At high concentrations even glucocorticoids can bind to PR and in the same fashion at high concentrations even progesterone binds to androgen receptors and glucocorticoid receptors (47). PRs exist in two isoforms PR_A and PR_B; in endometrium the ratio of these two isoforms vary constantly during menstrual cycle (48). Isoform PR_C also exists that is lesser known (49). Of the two isoforms, PR_B is a stronger transcriptional activator whereas transcriptional activity of PR_A is cell and gene specific (50). Inhibition of decidualization is seen in a PR_A knockout mice, suggesting an important role of PR_A in decidualization (51) whereas uterine responses to progesterone are not affected in a PR_B knockout mice (52).

1.5.1.1.3 Human Chorionic Gonadotropin (hCG)

hCG is the first confirmed marker of human trophoblast cells and is composed of α and β subunits that are non-covalently linked together. It is secreted by cytotrophoblast cells and induces extravillous cytotrophoblasts proliferation and invasion by inhibiting TGF-β receptors, thus preventing the apoptosis of trophoblast cells (53). Presence of hCG is the principal detection method in the pregnancy confirmation tests. hCG maintains the pregnancy, until the placenta takes over. hCG acts through luteinizing hormone/choriogonadotropin receptor that is present on the corpus luteum. Apart from this, hCG plays an important role in angiogenesis, decidualization, immune modulation and remodeling of extracellular matrix in the endometrium.

1.5.1.1.4 Corticotropin releasing hormone (CRH)

CRH is a neuropeptide that is secreted from the hypothalamus in response to stress reaction and is a principal regulator of Hypothalamo-Pituitary Axis (HPA). Endometrium during blastocyst implantation shows characteristics of acute inflammatory response from the invading semi-allograft blastocyst; however once implantation is successful, the embryo suppresses this reaction. CRH also plays a critical role on the maternal immune system to prevent graft vs. host reaction by slaying of activated T cells through Fas-Fas Ligand interactions. Blockage of CRH receptors by an antagonist antalarmin, reduces the implantation sites by 70% in animal models. On the other hand, *in vitro*, CRH receptor blockade increases the trophoblast invasion by 60% suggesting a role in regulation of trophoblast invasion (54). A defective CRH/CRH receptor system is usually seen in recurrent implantation failure (RIF) patients, placental defects and preeclampsia conditions.

Intrauterine administration of autologous CRH-treated peripheral blood mononuclear cells increased the positive pregnancy by 44% in RIF patients, suggesting a potential role for CRH in treating the RIF patients (55).

1.5.1.1.5 Calcitonin

Calcitonin / thyrocalcitonin is a known potential regulator of implantation and a marker of endometrial receptivity (56, 57). It plays a critical role in calcium homeostasis in the body by reducing serum calcium concentration in response to hypercalcemia and also acts counter to the parathyroid hormone. During WOI a high concentration of calcitonin in endometrium is seen (58) and inhibition of calcitonin synthesis by antagonists in mice reduces the implantation rates by 50-80% (56). It is hypothesized that calcitonin upregulates the expression of integrin β3 in endometrial epithelial cells facilitating implantation (59). *In vitro*, incubation of blastocysts with 10nM calcitonin has been shown to accelerate differentiation of blastocyst cells suggesting a role in embryonic development (60). Expression of calcitonin is regulated by progesterone and progesterone through calcitonin modulates the expression of E-Cadherins.

1.5.1.1.6 Leptin

Leptin, known as satiety hormone, is secreted from adipose cells and is encoded by *ob* gene. It plays an important role in endometrial receptivity and implantation by down-regulating γ-ENaC (Epithelial sodium channels) by activating STAT3 signalling pathways (61). ENaCs play a key role in regulating endometrial receptivity and an altered expression has been shown to cause impaired endometrial receptivity and implantation failures (62). High levels of leptin are seen in patients with recurrent miscarriage and RIF (63).

1.5.1.2 Pinopodes

Pinopodes are typical bleb like protrusions on the uterine luminal epithelial apical surface that appear during the window of receptivity (64). Role of ovarian hormones in the development of pinopodes is variable; appearance of pinopodes is strictly progesterone dependent whereas supra physiological levels of estrogens cause loss of pinopodes and physiological levels of estrogen favor the pinopode formation (65). The exact role of pinopodes remains unknown; however pinopodes seem to have various roles in blastocyst implantation that include mediation of endocytosis, exchange of fluids and facilitate the adhesion of blastocyst. It has been shown that pinopodes are the preferred site for blastocyst attachment (66). Pinopodes also release secretory vesicles that are rich in LIF (67), an important cytokine which influences endometrial receptivity by regulating trophoblast function and vascular formation in placenta.

1.5.1.3 Mucins

Mucins form a protective biofilm on the surface of endometrial epithelium and also maintain local molecular microenvironment (68). MUC1, MUC16 and to a lesser extent MUC6 have been shown to be expressed in endometrium among all the cloned human mucins (69-71).

MUC1 also known as Episialin/ Epithelial Membrane Antigen, is synthesized in rough endoplasmic reticulum and becomes glycosylated in golgi apparatus (72). DNA Methylation studies reveal that MUC1 gene is regulated by DNA methylation and Histone H3 Lysine9 (H3-K9) modification at the MUC1 promoter site (73). Significant reduction of MUC1 expression is observed in women with recurrent pregnancy loss (74). MUC16 a component of non-receptive luminal epithelium, prevents trophoblast adhesion and during the implantation at the time of pinopode formation, MUC16 is eliminated favoring adhesion of trophoblast cells (71).

1.5.1.4 Cell adhesion molecules (CAMs)

CAMs are transmembrane receptors that are mainly involved in cell adhesion. CAMs are composed of three domains, an intracellular domain, an extracellular domain and a transmembrane domain. CAMs are subdivided into 4 groups namely Cadherins, Integrins, Selectins and Immunoglobulin superfamily (IgSF). Cadherins and selectins are calcium dependent for execution of their function whereas IgSF and Integrins are calcium independent. Apart from the mentioned CAMs, Trophinin in combination with tastin and bystin plays an important role in endometrial receptivity and implantation.

1.5.1.4.1 Cadherins

Cadherins are type-1 transmembrane glycoproteins and are calcium ion dependent to serve their function. Primary function of cadherins is formation of adherens junction to promote cell-to-cell adhesion, also involved in tissue structure maintenance and in cellular movement. Cadherins are classified into Epithelial-Cadherins (E-Cadherin), Placental-Cadherins (P-Cadherins) and Neural-Cadherins (N-Cadherin), which are tissue specific (75). E-Cadherin is the most studied cadherin in endometrial receptivity and embryo implantation.

E-cadherins are located in the adherens junctions on lateral side of epithelial plasma membrane that are critical in maintenance of these junctions (76). Expression of E-cadherins is high during the luteal phase (77). Precise role of E-Cadherin in human implantation is unknown, however any mutations in E-Cadherin gene lead to a defective preimplantation development in mice (78). E-Cadherins exhibit a dual role in endometrium. In pre-implantation period, E-Cadherins increase the adhesiveness of the epithelial cells and avoid the implantation outside the WOI period. During implantation period a rise in progesterone increases the intracellular calcium ions mediated by the increase in endometrial calcitonin hormone expression, in turn the increased calcitonin decreases the expression of E-Cadherins making the epithelial cells less adhesive and enables epithelial cell dissociation facilitating the embryo implantation process (58, 79).

Sirtuin 1 (SIRT1), a class III histone deacetylase, *in vitro* has been shown to improve implantation rates that are mediated by an increase in the expression of E-cadherins in a dose dependent manner. SIRT1 stimulating drugs such as resveratrol in *in vitro* experiments on cell lines using embryo implantation models demonstrated an improved implantation rate, the repressors of SIRT1 decreased the implantation by reducing E-cadherin expression (80). This indicates that the mentioned chemicals could be used as therapeutic targets for improving implantation process and a success in ART can be achieved, but with a caution that the pathophysiology of implantation varies between *in vitro* and *in vivo* and it would be interesting to explore this hypothesis in *in vivo* models.

1.5.1.4.2 Integrins

Integrins are family of transmembrane glycoproteins whose primary function is attachment of cell to extracellular matrix (ECM) and signal transduction from ECM to cell. αvβ3 integrin and its ligand osteopontin are the most studied of all integrins in the context of endometrial receptivity and implantation. αvβ3 is a receptor for vitronectin and is composed of integrin alpha V and integrin beta 3/ CD61. Immunohistochemical detection techniques show that αvβ3 and osteopontin are located in endometrial luminal epithelial surface that first interacts with the blastocyst (81). Receptors for integrins are also expressed on the blastocyst during implantation window (82). Expression of integrins is seen highest during the implantation window and a blockade of αvβ3 receptors results in decreased number of

implantation sites in a mice model whereas in rabbit models implantation is inhibited (83, 84).

In a clinical trial, Integrins β1 and β3 along with serum estrogen and progesterone levels are suggested to be good biomarkers for determining optimal time for ET and in assessing endometrial receptivity (85). During implantation integrins β1 and β3 disassemble from site of focal adhesions and cause removal of luminal endometrial epithelial cells to facilitate implantation (86). Apart from αvβ3, integrins such as Integrin β8 (87), αvβ5 (88) have an essential role in implantation process. Interestingly, in women with impaired fertility, expression of integrins α4β1, αVβ1 were upregulated in glandular epithelium and stroma (89) and in recurrent pregnancy loss a significant reduction of integrin expression is observed (90).

1.5.1.4.3 Selectins

Selectins are a family of heterophilic CAMs i.e. binding of extracellular domain of CAMs to ECM, that binds to mucins. Selectins are classified as Endothelial selectins (E-Selectins), Leukocyte selectins (L-Selectins) and Platelet selectins (P-Selectins). Selectins play an important role in leucocyte transendothelial trafficking (91). The L-Selectin adhesion system i.e. L-Selectins and its oligosaccharide ligand MECA-79 plays an important role in implantation and is considered as most important pathway for embryo-endometrial interactions. L-Selectin receptors are expressed on the surface of blastocyst and the expression in endometrium is seen during the mid secretory phase particularly in pinopodes (92) suggesting a role in implantation.

MECA-79 is predominantly present in the glandular compartment and seen throughout the menstrual cycle. However, an increased expression is seen during the mid secretory phase i.e. during WOI (93). Lack of MECA-79 expression during the mid secretory phase is seen in women with recurrent implantation failure (94). Higher expression of L-Selectin ligand is associated with an improved pregnancy outcome in women undergoing IVF and ET (95).

1.5.1.4.4 Immunoglobulin superfamily (IgSF)

Intercellular adhesion molecule-1 (ICAM-1)/CD54 is a transmembrane glycoprotein expressed on various cells such as fibroblasts, leukocytes, endothelial and epithelial cells. ICAM-1 contains a ligand for widely expressed integrin β2 and mediates cell adhesion, which is essential for different immunological functions such as natural killer cell mediated cytotoxicity and transendothelial migration of leukocytes (96). ICAM-1 is expressed throughout menstrual cycle at the apical surface of epithelial glands and stroma, whereas

upregulation in the expression is seen during the menstruation in stromal cells and a significantly reduced expression is seen in secretory endometrium of endometriosis patients (97).

In serum and peritoneal fluid a circulating soluble form of ICAM-1 (sICAM-1) is seen as a result of shedding of transmembrane bound ICAM-1. Interferon-γ (IFN-γ) upregulates the expression of ICAM-1 in the endometrial stromal cell cultures and also allows the accumulation of sICAM-1 (98). Stromal cells of eutopic endometrium from women with endometriosis, when stimulated with IFN-γ, display an upregulation of sICAM-1. Altered expression of vascular CAM-1 (VCAM-1) and ICAM-1 are observed in women with endometriosis and ratio of soluble VCAM-1/ sICAM-1 is a promising biomarker for diagnosing endometriosis non-invasively (99). Higher levels of tissue ICAM-1 is seen in patients with recurrent pregnancy loss (100) and ICAM-1 plays an important role in endometriosis pathogenesis by facilitating escape from immunosurveillance, of the refluxed endometrial cells and allows them to spread and invade other locations (98).

Basigin / CD147/ EMMPRIN is a member of IgSF. It exists as component of receptor complex on trophoblast and is involved in the regulation of implantation, invasion and differentiation of trophoblast cells (101). Knockout of *BSG* gene leads to infertility (102).

1.5.1.4.5 Trophinin – Tastin – Bystin complex

Trophinin is a CAM that is involved in initial attachment of embryo in the implantation process. In addition it mediates the cell adhesion between trophoblast cells and luminal epithelial cells. Peak expression is seen during the mid luteal phase and expression is also seen in trophectoderm cells of embryo and placenta.

Trophinin in combination with tastin and bystin forms a complex that mediates the unique adhesion of embryo to luminal epithelium (103). It is hypothesized that cell adhesion mediated by trophinin induces the apoptosis of epithelial cells to facilitate invasion by trophoblast cells (104). Decreased expression of trophinin leads to decreased implantation rates, as observed in endometriosis patients and infertile women (105, 106).

Bystin and Tastin are cytoplasmic proteins that are expressed along with trophinin in trophoblast cells and endometrial epithelial cells at the site of implantation. Exact mechanism of action of this complex on endometrial receptivity and implantation is unclear and further studies on this complex provide more detailed insights into the molecular mechanism of implantation and may contribute to progress in reproductive medicine.

1.5.1.5 Cytokines

Cytokines are group of small proteins (5-20kDa) that play an important role in cell signaling. Cytokines act as immunomodulating agents that are involved in autocrine, endocrine and paracrine signalling. They are produced by a broad range of cells in the body and are involved in multiple cell functions like proliferation, differentiation, play a key role in immune system, role in menstrual cycle and are also critical for implantation.

1.5.1.5.1 Leukemia inhibitory factor (LIF)

LIF is an Interleukin 6 class pro-inflammatory cytokine that play an important role in cell differentiation. It is normally expressed in trophectoderm of embryo and its receptor (LIFR-α/LIFR) is expressed throughout the inner cell mas in the blastocyst. In endometrium its expression is seen in the glandular compartment and is proven to be an important and first cytokine factor to be involved in the regulation of uterine receptivity.

LIF binds to its receptor LIFR and forms a complex with gp130, a signal transducing subunit and a common receptor for IL-6, i.e. LIF-LIFR-gp130 complex, leading to activation of JAK/STAT and MAPK cascades in the epithelial cells and specifically luminal cells. The cascade of changes includes change in the epithelial polarity, angiogenesis, epithelial-mesenchymal interactions, decidualization in stromal cells and inhibition of cell proliferation. Apart from the activation of JAK/STAT pathways, it also activates many important signalling pathways which are essential for implantation such as TGFβ signalling, FGF signalling, VEGF signalling, Wnt-β catenin signalling, PTEN signalling and Notch signalling.

Progesterone plays a significant role in regulation of LIF and administration of anti-progestin mifepristone after the day of ovulation is shown to reduce glandular LIF (107). LIF and LIFR play a critical role in various endometrial pathologies and diseases affecting fertility. In adenomyosis, a significant reduction in LIFR expression is seen, which reduces the STAT3 signalling pathways mediated by LIF and leads to declined implantation rates in women (108). Similar abnormal levels of LIF are seen in women with unexplained fertility and RIF patients. Evidence of LIF and gp130 secretions in the uterine fluid opens a novel way for diagnosis of endometrial receptivity non-invasively that helps in predicting successful implantation in women treated with recombinant LIF (109).

1.5.1.5.2 Interleukin-1 (IL-1)

IL-1 cytokine family is a group of 11 cytokines and are key mediators of immune and inflammatory responses. In IL-1 knockout mice, which are fertile, an intraperitoneal injection

of IL-1R antagonist prevented blastocyst implantation by reducing the integrin expression in luminal epithelial cells (110), a similar phenomenon that is also seen in humans. An increased expression of integrin β3 is seen in the endometrial epithelial cell cultures supplemented with IL-1, a similar function is seen for IL-1β, which is mediated by secretion of leptin from epithelial cells (111, 112). *In vitro*, treatment of endometrial 3D cell cultures with mifepristone reduces the expression of IL-1 suggesting a regulation by progesterone (113).

1.5.1.5.3 Interleukin-6 (IL-6)

IL-6 is a pro-inflammatory cytokine as well as anti-inflammatory myokine that act through IL-6R and gp130 receptors. Expression of IL-6 & IL-6R is highest in mid to late secretory phase, strongest expression seen in the epithelial compartment (114). A predicted paracrine or autocrine role for IL-6 in implantation and endometrial receptivity is suggested since receptors for IL-6 are expressed by blastocyst and endometrium. Reduced sites of implantation and fertility were seen in IL-6 deficient mice, however disruption of IL-6 gene in mice did not affect the blastocyst implantation but the blastocysts were underdeveloped (115).

1.5.1.6 Growth factors

Growth factors are naturally occurring cell derived polypeptides that act as signalling molecules and stimulate the growth of the cell. Expression of receptors for various growth factors on the endometrium suggests an important role in maintaining endometrial receptivity and implantation.

1.5.1.6.1 Transforming growth factor-β (TGF-β)

TGF-β plays a significant role in tissue remodeling and reproductive processes and exists in TGF-β1, β2 and β3 isoforms. TGF-β1 & TGF-β3 are predominantly localized in both the epithelial and the stromal compartment whereas TGF-β2 is seen only in the stroma. Abundant expression of TGF-β in the endometrium suggests an active role in modulating the cellular events responsible for menstruation, decidualization, implantation and maintenance of pregnancy (116). TGF-β regulates endometrial remodeling in each menstrual cycle by acting through PI3K/AKT survival pathway along with inhibition of XIAP, an anti-apoptotic protein (117). Gargett et.al have demonstrated in cell cultures that inhibition of TGF-β receptor signalling promotes and maintains the stemness of endometrial MSCs (118).

TGF-β was shown to play a critical role in establishment and progression of various endometrial pathologies such as intra uterine adhesions, endometriosis, heavy menstrual bleedings and adenomyosis (119-121).

1.5.1.6.2 Insulin like growth factor (IGFs)

IGFs are proteins that have high similarity in sequence to insulin that form a part of complex network in cell communications. IGFs bind to IGF Receptors (IGF1R and IGF2R) on cell surfaces for mediating their biological activity. IGFs consist of two ligands IGF1 and IGF2. High expression of IGF1 is seen during the peri-implantation period and is assumed to be involved in the decidualization process (122) and enhances embryo development and quality. IGF2 is a known mediator of trophoblast function (123) and its expression in blood vessels near the implantation site suggests a role in angiogenesis (124).

1.5.1.6.3 Colony stimulating factors (CSFs)

CSFs include CSF1, CSF2 and CSF3. CSF1/ macrophage CSF (M-CSF) is a secreted cytokine that binds to CSF1 receptor (CSF-1R). It is presumed to be involved in development of placenta and decreased implantation rates are observed with mutation of CSF gene (125, 126). Decreased mRNA concentration of CSF1 is observed in patients with recurrent miscarriage (127) whereas an increased expression is documented in early endometriotic lesions or endometriosis (128, 129).

CSF2/ Granulocyte-Macrophage CSF (GM-CSF) is a monomeric glycoprotein secreted by NK cells, T-cells, endothelial cells, macrophages and fibroblasts. It is a potent inducer and activator of both macrophages and dendritic cells that play a crucial role in preeclampsia (130). Insufficient CSF2 leads to impaired generation of T-cell mediated immunity at the onset of pregnancy, which may lead to infertility, miscarriage or pre-eclampsia (131).

CSF3/ Granulocyte-CSF (G-CSF/GCSF), a glycoprotein that stimulates bone marrow to produce stem cells and granulocytes. An increased implantation rate and positive clinical pregnancies are reported with transvaginal perfusion of GCSF in IVF undergoing woman with thin endometrium or repeated IVF failures (132-134), however exact mechanism of action is unknown.

1.5.1.6.4 Heparin binding –epidermal like growth factor (HB-EGF)

It is a member of epidermal growth factor family that interacts with EGF receptor and TGF-α receptor. Expression is seen throughout the menstrual cycle, however increased expression is seen during the secretory phase and with particularly highest expression prior to implantation (135). Co-existence of HB-EGF in the glandular compartment and pinopodes at same time suggests an important role in attachment and invasion processes of implantation (136). Recently it is shown that HB-EGF stimulates EGFR/ERK signalling to promote Aquaporin 3 expression in trophoblast cells, which play a vital role in HB-EGF induced implantation (137). Reduced levels of HB-EGF protein expression is seen in mid secretory endometrium in women with unexplained infertility (138).

1.5.1.6.5 Keratinocyte growth factor (KGF)

KGF / FGF7 is usually observed in epithelialization phase of wound healing. KGF is induced by stromally derived progesterone and also acts as progestomedin (139). In *in vitro* cultures KGF acts as a mitogen for epithelial cells. Peak expression of KGF during WOI stimulates the spiral artery growth that is essential for successful implantation, in addition to inhibition of apoptosis of the glands (140).

1.5.1.7 Proteins

1.5.1.7.1 Glycodelin

Glycodelin/ Placental Protein 14 (PP14)/ Placental α2-macroglobulin/ progesterone dependent endometrial protein (PAEP) is a glycoprotein that has similar characteristics like integrin β3 and exists in three isoforms in amniotic fluid (Glycodelin-A), in seminal fluid (Glycodelin-S) and in follicular fluid (Glycodelin-F) (141). It plays a critical role in modulating maternal immune system to permit the fetal allograft by suppressing the cytolytic activity of CD8+ T lymphocytes (142). Increased expression of glycodelin is seen during the secretory phase and particularly during the WOI. Glycodelin-A is abundant in receptive endometrium (143) and also seen in the pinopodes of receptive endometrium. Clinically, reduced levels of glycodelin are observed in women with polycystic ovary syndrome (PCOS), endometriosis (144) and in RIF patients (145).

1.5.1.7.2 Annexin IV (ANX4)

ANX4 belongs to family of calcium-dependent phospholipid binding proteins and plays a role in the regulation of ion and water transport across epithelial cells in endometrium. Expression of ANX4 varies throughout the menstrual cycle, a weak expression is seen during the proliferative phase compared to mid and late secretory phase in glandular compartment. (146). Reduced ANX4 expression levels are observed in women with endometriosis suggesting a role in endometrial receptivity (147).

1.5.1.7.3 CD9 or Motility related protein-1 (MRP1)

CD9 / MRP1 is considered as one of the stem cell markers expressed in the epithelial glands. CD9 forms a complex with integrin α3 at cell contact sites and mediates integrin signalling in different processes such as cell proliferation, differentiation, motility and adhesions (148). Expression status of CD9 and integrin α3 provides valuable insights to the behavior of endometrial cancers; an aggressive behavior of endometrial tumors is observed in lack of CD9/MRP1 co-operation with integrins (149). CD9 regulates the

embryo invasion during implantation by acting through PI3K signalling (150) and severe fertility reduction is seen in CD9 deficient mice (151).

1.5.1.7.4 CD98

CD98 is a glycoprotein that is involved in amino acid transport and cell fusion. It forms multiple complexes with CD147, integrins β1, β3 and ICAM1. Its expression in epithelial cells is strictly restricted to implantation window in a hormone dependent fashion. Lack of CD98 affects the blastocyst adhesion (152) and lethal phenotypes resulted in mice with gene deletion for CD98 (153) suggesting that CD98 plays an important role in endometrial receptivity.

1.5.1.7.5 CD43

CD43 /Sialophorin/Leukosialin is a transmembrane cell surface protein that is localized in epithelial compartment during the time of implantation. It is involved in adhesion of blastocyst to luminal epithelial cells (154) and is regulated by combination of estrogen and progesterone (155).

1.5.1.7.6 CD52

CD52 or CAMPATH-1 antigen is a glycoprotein that is expressed on lymphocytes, sperms, epididymis and epithelial cells. In mouse endometrium, CD52 expression peaks during WOI and is expressed specifically in luminal epithelial cells. It plays a role in induction of ovulation that is stimulated by ovarian gonadotropic hormones and is associated with NKX2.2 pathways, which regulates expression of several cytokines including LIF (156).

1.5.1.7.7 CD82

CD82/ Metastasis suppressor/ KAI1 is a glycoprotein that has an anti-migratory function in tumor cells. A rise in expression of CD82 from proliferative to secretory is seen in human endometrium suggesting its regulation by progesterone. CD82 is localized to both the glandular and stromal compartment, being weakest in the stromal cells. It facilitates embryo adhesion to the endometrium through FAK signalling pathways (157). In addition, CD82 participates in intercellular communication with trophoblast cells thereby controlling trophoblast invasion (158). Altered levels of CD82 expression are observed in endometriosis patients implying a role in pathogenesis of endometriosis (159) and a decreased expression is seen in advanced endometrial cancers (160).

1.5.1.7.8 Galectins (Gal)

Galectins are a family of proteins that are lectins having galactose-binding ability in addition to amino acids. Till date 15 isoforms of galectins have been discovered, however galectins 1 and gal 3 are the ones that are widely studied in the endometrium and to a lesser extent gal 9 and gal12. Galectins have a role in modulating the endometrial immune system, cell adhesion, chemotaxis and protection of endometrium from invading pathogens. Gal-1 is localized in the stroma and seen throughout the menstrual cycle being up regulated in late secretory phase and early pregnancy decidua whereas Gal-3 is localized in the glandular epithelium and follows same pattern as gal-1 and highly expressed during WOI (161).

1.5.1.7.9 Osteopontin (OPN)

OPN/ Bone Sialoprotein 1 (BSP1) is a secreted phosphoprotein 1 (SPP1) expressed on luminal epithelial cells and is a ligand for integrin αVβ3. Integrin αVβ3 through its ligand OPN mediates the adhesion of embryo to the luminal epithelium during implantation. It regulates the expression of FoxM1 (Forkhead box M1), which in turn activates ERK 1/2, and p38 signalling pathways leading to proliferation of cells (162). Significant presence of Osteopontin-αVβ3 complex in implantation suggests this complex as a biomarker of endometrial receptivity (163). In women with endometriosis, a decreased expression during WOI is observed suggesting an important role in infertility (164).

1.5.1.7.10 Claudin 4 (CLDN4)

Claudin 4 is an integral membrane protein that forms a part of tight junctions in the cells to form aqueous and ion-selective pores. Interestingly, combined immunolabeling of $CLDN4^{-}/LIF^{+}$ could serve as a potential biomarker to predict successful pregnancy outcome in women undergoing IVF (165). CLDN4 plays a significant role in pathogenesis of endometriosis and a decreased expression is seen in the ectopic endometrium (166). It is proposed that CLDN4 could serve as a useful biomarker for monitoring treatment of endometrial carcinomas as an abnormally high expression of CLDN4 is observed in various endometrial carcinomas (167).

1.5.1.8 ENZYMES

1.5.1.8.1 Serum/Glucocorticoid regulated kinase 1 (SGK1)

SGK1 is induced by progesterone and plays a role in ion and solute transport through ENaCs in luminal epithelial cells. However, a decreased expression of SGK1 is seen during the WOI in the luminal epithelium and a rise in SGK1 levels during WOI is associated with inhibition of implantation in humans and mice (168), a similar observation is seen in women with unexplained infertility (169). Activation of SGK1 leads to inhibition of PI3K/AKT signalling pathways and activates ENaCs and other ion channels causing premature closure of receptivity, ultimately inhibiting embryo implantation (170). LEFTY-A, a known regulator of stemness and embryonic differentiation and a negative regulator of receptivity, has been shown that it activates and regulates the epithelial ENaCs acting through SGK1 thereby decreasing endometrial receptivity (171).

1.5.1.8.2 Matrix metalloproteinases (MMPs)

MMPs are calcium dependent zinc containing endopeptidases whose primary function is degradation of ECM proteins, cell proliferation, differentiation, apoptosis etc. MMPs and their inhibitors TIMPs play a critical role in implantation by ECM remodeling and are regulated by progesterone. Abnormal expression of MMPs is associated with disturbances in implantation and fertility. In women with impaired fertility, a high level of MMP2 expression is seen during WOI (172) and dysregulation of MMP2, MMP9 and TIMP1 results in infertility and early pregnancy loss (173). In addition, MMP2 promotes angiogenesis that is mediated through COX-2 and AKT signalling pathways and helps in endometriosis progression (174).

1.5.1.8.3 Fucosyltransferases (FUT)

Six members constitute this enzyme family (FUT3, FUT4, FUT5, FUT6, FUT7 and FUT9) that plays an important role in synthesis of fucosylated glycoconjugates that affect cell adhesion. In mice, FUT4, FUT7 and FUT9 show a peak expression during the day of implantation. In both *in vitro* and *in vivo* mice models, overexpression of FUT7 is shown to increase embryo adhesion and implantation rates (175). FUT4 via Wnt/beta-catenin signalling pathway promotes embryo adhesion and implantation and is considered as a general marker of endometrial receptivity (176).

1.5.1.8.4 Tissue transglutaminase (tTG)

tTG/TG2 is a calcium dependent enzyme of transglutaminase family. A ten fold higher concentration of tTG activity is seen during the luteal phase implying a progesterone

regulation (177). tTGs through their interaction with fibronectin network participate in the adhesion events of implantation process by activating integrins signalling pathways (178).

1.5.1.8.5 Monoamino oxidases (MAO)

MAOs are enzymes that catalyze oxidation of monoamines and are found in the outer membrane of mitochondria. MAOs exists in two forms MAO-A and MAO-B that are both expressed in uterus (179). MAO-A seems to play a role in endometrial receptivity as its increased expression appears in the receptive phase of the glandular endometrium, particularly mid-luteal phase. It is presumed that MAO-A promotes implantation process by inhibiting 5-hydroxytryptamine, a potential toxic molecule (180). Lack of MAO-A expression results in decreased endometrial receptivity in infertile women.

1.5.1.9 Transcription factors

1.5.1.9.1 HOXA-10/HOXA-11/Homeobox genes (Hox)

Hox genes encode proteins that are essential for embryonic morphogenesis and differentiation. Expression of HOXA-10 & HOXA-11 is observed in both the glandular and the stromal compartment, peak expression is seen during WOI. Hox plays an important role in implantation and any mutations of these genes result in failed implantations and infertility (181). HOXA-10 is essential for development of proper female reproductive tract, stromal decidualization and pinopode development. Impaired uterine development is seen in HOXA-11 null mutant mice. In infertile women with intramural fibroids and endometriosis a reduced expression of HOXA-10 and HOXA-11 is seen (182).

1.5.1.9.2 Forkhead box protein O1 (FOXO-1)

FOXO-1 plays an important role in regulation of gluconeogenesis and glycogenolysis by insulin signalling. FOXO proteins regulate genes of proapoptosis, differentiation, DNA repair and are essential for progesterone receptors to bind target genes (183). FOXO1A is known for its critical role in decidualization. Overexpression of SGK1 decreases the transcription of FOXO1 and ultimately reduces the expression of prolactin, a key marker of decidualization (169). In addition, FOXO1 also increases IGFBP1 expression during decidualization by binding to insulin response element (IRE) in IGFBP1 promoter region. In PCOS patients a compensatory hyperinsulinemia is observed with unexplained infertility and it is hypothesized that insulin in a dose dependent manner decreases the expression of FOXO1 and its target genes affecting decidualization (184).

1.5.1.9.3 Chicken ovalbumin upstream promoter transcription factor 2 (COUP-TF 2)

COUP-TF 2/ Nuclear receptor subfamily 2, group F, member 2 (NR2F2) is a protein encoded by *NR2F2* gene. A high expression is seen in stroma and is regulated by progesterone-IHH (Indian Hedge Hog) signalling pathway, which is secreted from the epithelium. Conditional knockout of COUP-TF2 in mice is shown to be associated with infertility and impairment of implantation and decidualization, suggesting a very important role in endometrial receptivity. *In vivo*, epithelial progesterone induces IHH that regulates COUPTF2 in the stromal cells, in turn COUPTF2 acts through its downstream target BMP2 (Bone morphogenetic protein 2) that successfully establishes decidualization and implantation (185).

1.5.1.9.4 Heart and neural crest derivatives-expressed protein 2 (HAND2)

HAND2 is a transcription factor regulated by progesterone and expressed in stromal cells. Selective HAND2 knockout mice lack the capability for embryo implantation, suggesting an important role in endometrial receptivity and implantation. Progesterone during secretory phase induces HAND2 expression in stromal cells, which in turn suppresses the production of various fibroblast growth factors (FGFs) that mediate the mitogenic effects of estrogens in epithelial cells. With lack of HAND2, as seen with anti progestin treatment, the mitogenic effect of FGFs are unopposed leading to proliferation of epithelial cells resulting in impaired implantation. Thus HAND2 plays a critical role in the cross talk between stromal-epithelial cells in order to establish a successful pregnancy by modulating endometrial receptivity (186). Epigenome wide methylation studies in endometrial cancers reveal that HAND2 is commonly hypermethylated and silenced, thus suggesting HAND2 as a biomarker for early detection of endometrial cancer (187).

1.5.1.10 Prostaglandins

Prostaglandins (PGs) are a group of physiologically active lipid compounds found in almost all tissues. PGs contain four members PGD_2, PGE_2, $PGF_{2\alpha}$ & Prostacyclin (PGI_2), metabolites of arachidonic acid produced by enzymes cytosolic Phospholipase A_2 ($cPLA_2$) and cyclooxygenase (COX-1, COX-2). Lack of $cPLA_2$ or COX-2 enzyme leads to several implantation defects due to lack of PGs (188). Most of the PGs are involved in implantation process and very little is known about their role in endometrial receptivity. Various roles of PGs in endometrium include proliferation of glandular epithelial cells during proliferative phase, vascular functions and role in menstruation. Vilella et.al have demonstrated that presence of PGE_2 and $PGF_{2\alpha}$ synthases in epithelial cells and concentration of these

substances in uterine fluid before 24hours of ET serves as potential non-invasive endometrial receptivity biomarker (189).

1.5.1.11 Fatty acid binding protein 4 (FABP4)

FABP4 is considered as a regulator of proliferation, migration and invasion of epithelial cells in endometrium (190). Estrogen upregulates expression of FABP4 and further upregulated in combination with progesterone. Significant reduction in the number of implanted embryos, decreased deciduas formation with pregnancy loss, inhibition of various endometrial receptivity factors are seen with FABP4 inhibition suggesting an important role in the regulation of embryo implantation by altering endometrial receptivity (191).

1.5.1.12 micro-RNAs (miRNA/miR)

miRNA are small non-coding RNA with around 22 nucleotides. Role of miR's in the endometrial receptivity and implantation are explored with the advanced technologies in the field of omics and many miR's such as miR-302a, miR-543, miR-125b are shown to be having a role in implantation and endometrial receptivity (192-194). mir-133b has been shown play an essential role in endometrial receptivity by reducing SGK1 expression and promoting the action of HOXA-10 in successful implantation (195). Lack of miR-451 in mice leads to decreased number of embryo implantations by acting on the target gene *Ankrd46* (196).

Interestingly, a higher level of miR-645 expression seen in the media of embryos with poor pregnancy outcome by IVF treatment and undetectable from media of healthy embryos indicates that miRNA are incorporated and used by developing embryos (197). Various ongoing studies have demonstrated the significance of different miRNAs and their role in embryo development, in early abnormal pregnancies and in implantation failures. In addition miRNA are shown to be valuable in predicting the IVF outcome.

1.5.2 Diagnosis of endometrial receptivity

Till date no proven clinical diagnostic test is available for determining the endometrial receptivity prior to infertility treatment or to assess the receptive status of endometrium before ET in an IVF cycle. Such tests, if available, could help us to identify receptive endometrium and also can be useful in personalized embryo transfer in RIF women. So far, such a diagnostic test that is clinically validated and universally accepted is not available for routine use.

Conventionally Noyes criteria is used to assess endometrial receptivity in a clinical setting. In addition to Noyes criteria, pinopodes, various biochemical markers such as MUC1, LIF, HOXA-10, COX-2 etc. are proposed for diagnostic purposes, but none of them have been validated and there has been no further success.

In a natural cycle with sample size of 62 endometrial biopsies, five genes are significantly upregulated during the WOI – LAMB3 (laminin beta3), ANGPTL1 (angiopoietin-like 1), NLF2 (nuclear localized factor 2), MFAP5 (microfibril-associated protein 5) and EG-VEGF (endocrine gland-derived vascular endothelial growth factor). These are proposed as new biomarkers for receptivity that could be tested in the endometrial pathologies and could be used as predictors of IVF outcome (198).

E-tegrity test, a method of testing endometrial receptivity based on the expression of integrin β3 in endometrial biopsies collected during the WOI (199) has been shown to be useful in assessing receptivity in fertility disorders such as endometriosis, hydrosalpinx etc. Integrin β3 plays a role in receptivity and reduced expression are seen in various endometrial pathologies (200).

Endometrial function test (EFT), a method in which Cyclin E, a cell cycle regulator and associated with glandular development, is assessed by immunohistochemical technique in the endometrial biopsies. Overexpression of Cyclin E is observed in abnormally developing endometrium and in infertile women (201).

Based on the transcription profile of the endometrium in various phases of pre-receptive phase of menstrual cycle, a customized genomic tool Endometrial Receptivity Array (ERA) comprising of 238 selected genes was introduced for detecting endometrial receptivity by next generation sequencing for clinical purposes (202). ERA is considered as the most accurate and objective test available today for assessing endometrial receptivity. Moreover, ERA displayed a high correlation sensitivity of 0.99758 and specificity of 0.8857 for endometrial dating, and predicted a specificity of 0.157 and sensitivity of 0.995 for pathological classification. A similar approach of using microarray based expression consisting of 126 genes, 126 gene model, was explored for the use in endometrial disorders (203) and an overlap of 61 genes which were common for both the datasets is observed.

Very recently, ER Map (Endometrial Receptivity Map) has been introduced which is a new personalized and molecular tool test to detect the uterine receptivity during WOI (204, 205). In ER Map a panel of 48 genes that are involved in endometrial proliferation and immune responses are analyzed by qRT-PCR method. By using discriminant model, ER Map classified 100% cases correctly to both fertile and infertile woman. An added advantage of ER Map over ERA is that the analysis is performed by qRT-PCR that is easily accessible and available in most clinical settings today.

However, it has to be noted that these techniques are expensive, time consuming and lack accuracy and low predictability in diagnosing receptive endometrium. Further, large clinical studies have to be done for proving all array based expression test if it is clinically useful and validation has to be done to accept them as a diagnostic test for endometrial receptivity.

1.5.3 Antiprogestins/ Drugs/ Contraceptives to study hormonal regulation of endometrial receptivity

Progesterone is the key steroid hormone driving the endometrial receptivity. Any drugs or substances that affect progesterone synthesis or block its action during the luteal phase disrupt endometrial receptivity and cause implantation failure and these can also be used as important tools in studying endometrial receptivity.

1.5.3.1 Levonorgestrel (LNG)

LNG is the most widely used drug for emergency contraception (EC) and is effective within 72 to 96 hours of unprotected sexual intercourse and works only before pregnancy has occurred. It is used as oral contraceptive pills since 1980s and in intrauterine contraception since the 1990s and is on the list of WHO's essential medicine list. LNG dosage for EC is a single dose of 1.5mg (or two 0.75mg doses 12hrs apart)(206, 207).

1.5.3.2 Antiprogestins / Selective progesterone receptor modulators (SPRMs)

Antiprogestins / Antiprogestogens / Progesterone antagonists / Progesterone blockers/ Progesterone receptor modulators (PRMs)/ selective PRM (SPRMs) are agents or drugs that bind to progesterone receptor and block the biological actions of progesterone. PRMs are commonly used for medical abortion and emergency contraception as well as for treating uterine pathologies such as uterine fibroids.

SPRMs are the agents that act on PRs as agonists or antagonist, the actions may vary based on tissue i.e. may act as an agonist in one tissue and antagonist in other tissue. SPRMs

exert both agonist and antagonist effect on PRs and minimal interactions with other steroidal receptors.

1.5.3.2.1 Mifepristone

Mifepristone (RU-486) is a steroidal anti-progestin and anti-glucocorticoid, to a lesser extent anti-androgen and is the first antiprogestin to be discovered. It has a partial agonist in action in the absence of progesterone and a competitive PR antagonist in the presence of progesterone. It exhibits more than twice of progesterone's affinity towards PR, more than three times that of dexamethasone, ten times that of cortisol towards glucocorticoid receptors, and less than one-thirds of that of testosterone towards androgen receptors. Mifepristone does not bind to mineralocorticoid and estrogen receptors. The effects of mifepristone administration depend on the dosage and the timing during the menstrual cycle; a single high pre-ovulatory dose of 200-600 mg inhibits ovulation by affecting follicular development whereas a single pre-ovulatory dose of 10 mg delays ovulation by three to four days (208-210). Immediate post-ovulation (on LH+2) administration of 200 mg mifepristone inhibits endometrial development in the secretory phase, thus making the endometrium non receptive to the incoming blastocyst, while no effects are seen on ovarian steroid hormone production and menstrual cycle. In addition, a once-weekly dose of 5 mg inhibits the endometrial maturation without inhibiting ovulation (211, 212). Mifepristone also provides effective contraception at a daily doses of 2 to 10mg, by suppressing follicular development, blockage of LH surge and by inhibiting ovulation and endometrial receptivity as well as inducing amenorrhea. At a daily dose of 0.5mg, a mild delay of endometrial development and a reduction in glandular diameter is produced, but no significant changes are seen in the menstrual cycle duration, menstrual bleeding, ovulation and ovarian steroid hormone secretions and the contraceptive efficacy is insufficient (213-215). Mifepristone is highly effective and widely used for medical abortion, cervical ripening prior to surgical abortion, treatment of uterine fibroids and has shown to be effective in reducing endometriosis related pain (216).

1.5.3.2.2 Ulipristal acetate (UPA)

UPA/ CDB 2914 is a second generation PRM and is on the list of WHO's essential medicines for EC. UPA has partial agonistic as well as antagonistic effects on PRs. A single dose of 30mg within 120 hours of unprotected sexual intercourse is available as an emergency contraceptive pill and is approved in Europe since 2009 and by FDA in 2010. In addition, a 5mg/day UPA is used as preventive surgical treatment of uterine leiomyoma in North America and Europe. Furthermore it has been documented to reduce the size of uterine fibroids and control excessive bleeding resulting from fibroids with UPA treatment for 13 weeks (217-219). Clinical trials demonstrate that UPA is superior to standard LNG-EC in

terms of preventing unwanted pregnancies (220-222). UPA, similar to mifepristone, displays a dose dependent response on the endometrium with decrease in endometrial thickness and down regulation of PRs when exposed to higher doses. Exposure to UPA as a single dosage EC i.e. at 30mg showed no significant difference compared with placebo on endometrial maturation (223).

1.6. Embryo implantation

Implantation is initiated with the attachment of blastocyst to the receptive endometrium, a critical step in establishing a successful pregnancy. Implantation is one of the most complex and tightly orchestrated events between embryo and endometrium and the mechanism differs in each species.

A successful implantation requires endometrium in a receptive phase, regulated by steroid hormones, and a viable blastocyst. Trophectoderm layer, outermost layer of blastocyst, undergoes a series of maturation leading to a stage known as 'activated' that includes metabolic changes and the ability to interact with endometrial luminal epithelial cells to undergo epithelial-mesenchymal transition for invasion process. The endometrium undergoes a series of changes that includes predecidualization, decidualization and secretory changes for enabling implantation.

1.6.1 Embryo-endometrial Interactions

Initial step to facilitate implantation is to build a dialogue between the viable blastocyst and receptive endometrium, and this is achieved with the support of growth factors and hormones. The prerequisite for implantation is that the blastocyst has to reach a stage of activation. Wnt-β-catenin signaling is considered as one of the pathways that helps the blastocyst to reach this activation; inactivation of Wnt signaling pathway limits the blastocyst competency for implantation (224).

hCG secreted by the early embryo is one of the earliest embryonic signals that is sensed by the receptive endometrium. Clinically detectable levels of hCG are seen even before the implantation process starts, whereas mRNA for hCG is detectable at 2 cell blastocyst stage (225) and is detected in urine after 7-8 days of conception. In humans and other primates, immunization against hCG prevents pregnancy by affecting the embryo implantation and it is suggested that immunization against hCG could serve as a contraceptive that is reversible (226). Embryonic hCG has both direct paracrine effect on endometrium and indirect endocrine actions on corpus luteum to secrete progesterone. This

progesterone increases LIF secretion mediated by IL-4 and therefore controls the function of endometrium (227).

Pre-implantation embryo also secrets several cytokines such as TGFα, EGF, IGF 1&2, PDGF-A, IL-1 system (IL-1α and IL-1β) that take part in embryo-endometrial interactions or signalling processes. IL-1 system acting via integrins α4, αV, β3 transforms the endometrium and also induces leukocyte differentiation (228). Furthermore, IL-1 induces the expression of G-CSF and LIF that bind to preimplantation embryo and activate IGFBP1 that helps in the embryo invasion. IL-1 stimulates the secretion of MMP-2 & MMP-9 that are involved in softening of stromal compartment during implantation (229).

During the implantation process, hormones such as estrogen and progesterone, cytokines, growth factors, chemokines and various other factors such as MUC-1, cell adhesion molecules, Leptins, HOX genes, MMPs etc. play an important role in the embryo-endometrial dialogue, the role of each mentioned factors in implantation has been briefly explained in the biomarkers of endometrial receptivity.

1.6.2 Implantation process

Three phases are involved in the implantation process, apposition, adhesion and invasion. During apposition the cells in the blastocyst orient towards the surface of endometrium and form a loose connection between blastocyst and endometrium. A dialogue between the endometrium and the blastocyst is mediated during apposition by the cytokines-IL-1, IL-6, IL-11, LIF and chemokines such as CSF-1 and GM-CSF, which act in a bidirectional fashion and help to dock the blastocyst to endometrium. A change in the polarity is seen in the endometrial luminal epithelial cells, a key aspect, during the initial apposition phase. In an *in vitro* model, it has been demonstrated that MUC-1 through its ligands for L-Selectin receptors that are expressed on trophoblast cells mediates the apposition (230). In addition, maternal LIF and HOXA-10 also play an important role in the initial attachment through their appearance in pinopodes/uterodomes, although exact mechanism is not clearly known (231, 232).

Adhesion ensues the apposition phase and it is in this phase that the embryo attaches strongly to the endometrium. Cell adhesion molecules such as integrins mainly mediate this phase and trophoblast cells transmigrate the luminal epithelium and penetrate into the endometrium. Integrins such as αvβ3, β8, αvβ5, α4β1, αVβ1, Basigin (MMP inducer), Osteopontin, Trophinin-Tastin-Bystin complex play a critical role in adhesion by mediating cell-to-cell and cell-to-matrix interactions.

Trophoblast invasion is the final phase of implantation in which the blastocyst invades into the decidualized stromal compartment by crossing the anatomical barrier of endometrium such as the luminal epithelium layer and basement membrane layer. After

successfully invading into the endometrium, the trophoblast cells differentiate into syncytiotrophoblast cells and cytotrophoblast cells. The syncytiotrophoblast cells achieve further invasion deep into the endometrium beneath the decidual cells, secrete TNFα and various matrix metalloproteases such as MMP-9 to lyse the extracellular matrix and make the whole embryo to be embedded in the endometrium. Subsequently, the cytotrophoblast cells invade the complete endometrium, uterine blood vessels and sometimes to a part of myometrium that ultimately initiates the placenta formation.

1.6.3 Challenges in studying endometrial receptivity and implantation

Implantation is a tightly orchestrated event between the receptive endometrium and the embryo and implantation is species specific. Co-ordinated actions of both steroid hormones, estrogen and progesterone, is essential for endometrium to prepare for implantation. WOI is a very restricted period and varies in different species, such as 24 hours in mice and 4-5 days in women, which makes studying WOI difficult.

In view of ethical and technical constraints, it is difficult to study the mechanism of endometrial receptivity and implantation in *in vivo* and suitable models to study implantation are lacking. Much of the information on implantation is derived from animal experiments and *in vitro* cell culture models. However the animal models and *in vitro* culture models have their own limitations – failure to completely translate the information from these models to humans in understanding the mechanism of human embryo implantation.

Though most of the *in vitro* cell cultures give clues on implantation, they lack cell-to-cell interactions, cell to matrix interactions, limited number of cell phenotypes, and lack of paracrine interactions. Most of the *in vitro* model experiments have concentrated only on the initial interactions of embryo/ blastocyst with the epithelial cells. Major constraint with the organ explant culture is that they undergo necrosis within a short time in *in vitro* conditions and cannot exactly replicate *in vivo* mechanism. The lack of polarization of epithelial cells in organ culture, which is a pre requisite for implantation, is another major limitation. Similarly, in monolayer co-cultures of stromal or epithelial cells with blastocysts, flat and non-polarized cells without intercellular junctions are observed while growing on plastic surfaces. Moreover the cross talk between the stromal compartment and epithelial compartment cannot be established. Interestingly, it is demonstrated that the blastocyst can attach to any surface covered by epithelial cells of mesodermal origin (233).

1.6.4 Ethics and usage of embryos in research

A question of debate since the invention of IVF is the usage of embryo for research purposes. However, the Human embryo research panel of National institute of health (NIH) in 1994 proposed the usage of spare embryos for research purposes up to 14 days post fertilization (234). The same policy is in effect in Sweden for the purposes of embryo research. Extreme care has to be taken to prevent the exploitation of spare embryos for therapeutic usage as stem cells are becoming common in routine treatments. Once the expiry date for storing of embryos in the freezers is passed, it is advantageous to use the embryos for research that could benefit the human beings, rather than destroying them.

1.6.5 Alternatives of embryo and endometrium in reproductive research

It is always difficult to obtain endometrial biopsies from women and spare embryos for research purpose, as using embryos for research is illegal in many countries. In reproductive medicine point of view, research in implantation failures and recurrent miscarriages leading to infertility is hampered by the availability of human tissue from which primary cells can be isolated and limited access to embryos worldwide for experimentations. Therefore use of the alternatives such as cell lines that represent endometrium and embryos could be an advantage for studying the implantation process and various gynecological disorders *in vitro*. Globally there are over 300 embryonic cell lines representing the trophoblast cells that are isolated from the embryos, and two thirds of them are present in just five countries – USA, UK, Sweden, France and Spain. Availability of different cell lines for endometrium and trophoblast makes the researchers difficult to select the cell lines for a particular function. In this section a brief description of cell lines available as alternatives to endometrium and embryo and their particular functions are discussed.

1.6.5.1 Epithelial cell lines

Most of the endometrial epithelial equivalent cells lines are derived from endometrial adenocarcinoma condition such as Ishikawa cells, ECC-1, HEC-1A, HEC-1B and RL-95-2 (235). Luminal epithelium equivalent cell lines for receptive phase endometrium are EEC-1, Ishikawa cells and HES cell lines, whereas HEC-1A is used as substitute for luminal epithelium in non-receptive phase endometrium. Ishikawa cells and RL95-2 can be used as alternatives of glandular epithelium. For functional studying of adhesion property in implantation, HEC-1A is poorly adhesive whereas Ishikawa and RL95-2 are highly adhesive to the trophoblast cells. Ishikawa cells have mixed characteristics of both glandular and luminar epithelium and are considered a good model for studying endometrial function.

In general, primary endometrial epithelial cells in *in vivo* conditions exhibit receptors for estrogen, progesterone, androgen, hCG and luteinizing hormone. It is important to choose

the cell line with suitable receptors for studying the endocrine signalling pathways in implantation or endometrial receptivity. Ishikawa cell lines possess receptors for estrogen, progesterone, androgens and luteinizing hormone whereas ECC-1 exhibits receptors for estrogen, progesterone and androgens. HEC-1A demonstrates receptors for estrogen and progesterone, on the other hand HEC-1B and HES only have estrogen receptors. RL95-2 cells have estrogen receptors and presence of receptors for progesterone is conflicting.

1.6.5.2 Trophoblast cell lines

Generally, the trophoblast cells are epithelial in nature and Keratin 7 (KRT7) is considered as positive marker of trophoblastic lineage *in vivo* that include cells such as syncytiotrophoblast cells, villous and extravillous cytotrophoblast cells and trophectoderm cells. Cell lines that are equivalent to trophoblasts include BeWo, AC1M-88, HTR-8/Svneo, JEG3 and JAR cell lines. Of these cell lines, BeWo cells are used for studying syncytialization, whereas for studying trophoblast adhesion/migration, AC1M-88 and HTR-8/Svneo cell lines are employed. For demonstrating invasion mechanism of implantation, HTR-8/Svneo, JEG3, BeWo and JAR cell lines are suitable.

1.6.6 Approaches to study endometrial receptivity and implantation

Ethical constraints limit the ability to study endometrial receptivity and implantation in humans. Till date numerous methods were used for studying endometrial receptivity and implantation in a quest to promote implantation ultimately resulting in a successful pregnancy. An important objective for research in the endometrial receptivity is to improve the current ART, since the success rate of ART is still around 30-40%, and in addition, to control the fertility by developing novels contraceptives. Advanced omics technologies such as sequencing, proteomics, transcriptomics, lipidomics, secretomics, and metabolomics etc. have opened up new approaches in the field of biomarker discovery. The following approaches available as of now in the field of receptivity discovery are discussed briefly.

1.6.6.1 Morphological dating / Histological evaluation of endometrium

Till today a common technique that is used routinely in the field of endometrial physiology and pathology to assess receptivity is the criteria of morphological dating of endometrium published by Noyes et al (25). An improved version of morphological dating superior and reliable to Noyes criteria is the morphometric analysis, proposed by Johannisson in 1982 (26). 17 morphological features are measured in the morphometric analysis, 11 features in glands and 6 in stromal cells. The main disadvantage of the

morphological method in evaluation of endometrial receptivity is that it is very subjective and could vary between the observers.

1.6.6.2 Pathophysiology of endometrium / *In vitro* fertilization

One of the best approaches to study endometrial receptivity or implantation is by observing the changes in various endometrial pathologies such as endometriosis, RIF, hydrosalpinx, and endometrial carcinomas leading to infertility. Studying the molecular alterations and biochemical expressions in these pathological conditions provides valuable information in understanding the receptivity. Various factors such as integrin αVβ3, ICAM-1, Trophinin, HOXA-10, COUP-TF2, cytokines LIF, IL6 etc. are altered in pathophysiology of endometrium. This has led to the discovery and understanding of the importance of these factors in endometrial receptivity and implantation.

IVF is one of the promising ART techniques so far in treating infertile couples and provides a good tool for studying endometrial receptivity and improving embryo implantation. Studying the effects of various factors such as hormones, cytokines, ovarian stimulations, new synthetic substances and drugs used in treatment that affects endometrial receptivity could predict the IVF outcome and vice versa. It is widely known that embryo secretes various factors such as cytokines, RNAs, miRNA's that modulate the endometrium towards a receptive phase. *In vitro* modifications of the embryo culturing medium and conditions could help us to understand how these modifications affect the endometrial receptivity and implantation.

1.6.6.3 Animal models

The process of implantation is species specific and is very different in cattle, pigs and rodents from human beings. The main difference lies in that the embryo in humans appears to have aggressive behavior in invading the decidual tissue and spiral arteries. A similar phenomenon of invasion and implantation is seen in non-human primates such as rhesus monkeys and baboons. However, in rodents, the mechanisms of embryo implantation and uterine receptivity are entirely different in terms of anatomy and reproductive physiology such as lack of menstruation.

1.6.6.4 Imaging Technologies / Ultrasonography

The most conventional, non-invasive way to clinically assess endometrial receptivity is by using ultrasonography that measures endometrial thickness, architecture and blood flow. Thickness of endometrium varies each day till the day of ovulation under the influence of estrogens and not so significant change in thickness is seen during the

luteal phase. Endometrial thickness is a question of debate; a meta-analysis of the relationship between thickness and IVF outcome concluded that the mean thickness is significantly higher in pregnant woman, however the difference in mean thickness of <1mm is considered may not be clinically significant (236). In murines, Liu et al showed that ultrasound in combination with molecular imaging with contrast agents to VEGFR2 may give superior information in assessing endometrial receptivity (237).

Color Doppler ultrasonography assesses the changes in menstrual cycle progression and can quantify the endometrial vascular blood flow. Better assessment of receptivity can be done if a three-dimensional ultrasonography approach is used.

1.6.6.5 Omics Approach

1.6.6.5.1 Genomics / Transcriptomics

In the recent years, after the completion of human genome project, several microarray, transcriptomics, lipidomics, secretomics, proteomics and metabolomics studies have been done to identify the important genes and/or proteins that could serve as biomarker of endometrial receptivity. In general, omics technology is a high throughput experiment where thousands of gene or protein expression can be determined in a given condition. One of the challenges of the omics technology is that the data it generates is so enormous, which is not used and analyzed to the full extent due to various reasons such as lack of proper guidelines in data analysis, difficulty in deriving conclusions, computing issues and scarcity of well trained bioinformaticians.

Several attempts have been made to elucidate gene expression profile of progesterone modulated receptive endometrium. With the development of microarrays there are at least four studies on human endometrial gene expression during the luteal phase (238-240). Although all of these studies used the same technology, the results differ due to experimental design and data analysis. The differences include the day of endometrial biopsy, phases of the menstrual cycle compared with, genomic variation between patients, and pooling or non-pooling the isolated RNA. Despite having a very careful experimental design and controlled experimental setup, the results may still vary due to the difference in cellular composition, mainly stromal and epithelial cells and the layer of endometrium obtained in the biopsy sample. Thus it is of importance to study the compartmentalized receptivity gene expression in women in well-timed and defined material in order to understand the endometrial receptivity better. Majority of the endometrial receptivity biomarkers known today were derived using these same omics approaches.

Microarrays and transcriptomics based expression profiling has its own limitations such as sensitivity and specificity of the probes used in specific platforms, lot of background noise and hence is becoming obsolete. Introduction of Next Generation

Sequencing (NGS) overcomes the difficulties of microarrays and is proven to be superior to the microarrays. NGS has the capability to sequence all the mRNA's present in the given sample and is very sensitive in detecting the genes that are rarely expressed or in low expression. Various transcript isoforms and absolute quantification of transcripts can be done which cannot be detected by conventional techniques. With the discovery of role of small non-coding RNAs, such as miRNA, piRNA, lncRNA etc. in endometrial receptivity, NGS serves as the best available sensitive technique today to profile the small RNAs and to identify new and novel RNAs that affect receptivity. Some limitations with NGS are that it is time consuming, the cost per sample is high, requires high performance computing and well-trained bioinformaticians. Because of all these limitations, as of now NGS is available only in a few selected laboratories, but is becoming widely popular in routine clinical use to diagnose various pathologic conditions.

1.6.6.5.2 Proteomics

In comparison to genomics, proteomics provides more biological and relevant information since it is the end product of genes or RNA or DNA. However, proteomics is complicated than genomics because most of the time genome in an organism is constant whereas proteome differs from time to time and cell-to-cell, sometimes the mRNA may not be translated into protein or less translated due to rapid degradation of mRNA. In routine practice, the correlation between genomics and proteomics for the same sample is less, which maybe due to the several steps involved in the translation processes, various post translational modifications such as glycosylation, phosphorylation, ubiquitination, alternative splicing and variation in degradation properties of each protein (241). Traditionally proteins can be detected by various immunoassays and the current methodologies include 2D-DIGE (fluorescence 2-dimensional differential gel electrophoresis), mass spectrometry, reverse-phased protein microarrays and protein profiling. Proteomics provides an opportunity to discover the complex biological mechanisms involved in endometrial receptivity, fertilization, implantation and pregnancy.

1.6.6.5.3 Secretomics

Secretomics is a subset of proteomics in which all the secreted proteins from the cells or organs such as uterine fluids and embryo secretions are studied. Uterine fluid / uterine secretome provides an easy and mild non-invasive approach for sample collection and biomarker discovery by proteomic profiling. Uterine fluid proteomics perhaps is a best approach for receptivity biomarker discovery as it can be collected by aspiration or lavage in the same cycle of embryo transfer without a consequent injury or very minimal injury to the endometrium.

1.6.6.5.4 Metabolomics /Lipidomics

Metabolomics is the study and analysis of molecular metabolites in cells and fluids, which are the end products of gene expression. Metabolomics or lipidomics employs various techniques such as gas chromatography- mass spectrometry (GC-MS), High-pressure liquid chromatography (HPLC), Nuclear Magnetic Resonance (NMR), Near Infrared spectroscopy (NIR), liquid chromatography-tandem mass spectrometry (LC-MS/MS) etc. that can detect femtomolar quantities of metabolites with good reproducibility.

Lipidomics is an emerging, massive study of lipids existing in the cells or metabolic pathways. It is well known in the field of endometrial receptivity that lipid compounds such as prostaglandins that are derivatives of COX enzymes, lysophosphatidic acid (LPA), endocannabinoids and sphingolipids play a key role in receptivity and implantation. Lipidomics of endometrium could be studied by analyzing the uterine fluid. PGE2 and PGF2α significantly increase in concentration during WOI in the endometrial fluid collected at different stages of menstrual cycle (242).

1.6.6.5.5 Epigenomics

Epigenomics is the study of epigenetic changes on the genetic material of cell i.e. epigenome. Important types of epigenetic modifications include DNA methylation and histone modifications. Epigenomics could be studied by techniques such as histone modification assays, DNA methylations assay (Bisulfite sequencing), ChIP-Chip and ChIP seq. It is believed that the cyclical changes that are observed in the endometrium in each menstrual cycle are under epigenetic control.

1.6.6.5.6 miRNA/miRs

miRNAs are small non-coding RNAs that regulate the stability and translation of mRNA. Methods to study miRNAs include high throughput miRNA sequencing, miRNA arrays, miRNA microarrays, RTPCRs and Locked nucleic acid (LNA) methods. Unlike other omics techniques, studying miRNAs has to be extremely careful as it involves technical variables such as isolation methods and easy degradation of miRNAs in comparison to mRNA due to their length. In addition, ubiquitous presence of RNAses makes it difficult to study miRNAs.

Role of miRNAs in the endometrial receptivity and implantation are explored using advanced technologies. The first study on miRNA profiling that is published in 2011, using the advantage of deep sequencing and bioinformatics analysis, had revealed the expression of 626 miRNAs in natural and stimulated cycles of human endometrium (243).

1.6.6.6 Cell/ Tissue cultures

In vitro cell culture models provide the best and easy approach for studying the receptivity as well as *in vitro* implantation. Greatest advantage of cell cultures is that rather than using primary cells from the endometrium, choice of cell lines alternative to the human endometrium and embryos are available easily. In addition, cell/tissue cultures provide a choice for studying different synthetic compounds of interest, drugs, cytokines and effects of various contraceptives. However, we have to be cautious because implantation and many other biological processes differ *in vitro* and *in vivo*; moreover the metabolism of drugs and compounds differ *in vitro* and *in vivo*. Cell/ tissue cultures are further classified as explant organ cultures, conventional 2Dimensional models i.e. monolayer cell cultures and multilayer cell co-culture models such as 3Dimensional cell co-culture models.

In organ explant culture, organs or small explants or slices of the organ are dissected *in vivo* during surgeries or *in vitro* and are maintained for extended times. Advantage with organ explant cultures is that it can be used to study pathophysiological mechanisms of the cells *in vitro*, to characterize the histological relationship between various cells in the tissue or organ. Perfusion studies on whole uterus obtained by hysterectomy is employed to exploit the interactions of trophoblast cells and various test substances added to perfusion medium (244). In endometrial explants, the structure of endometrium is maintained *in vitro* and this model helps to analyze the synthesis and secretions of endometrial cells into medium to explore the paracrine interactions. Limitations of this model are the differential viability of the individual cells and injured or necrotic cells may modify the responses.

In monolayer cell co-cultures, cells isolated from the tissue or cell line of choice are seeded on a plastic surface and the interactions their interactions with embryos or trophoblast cell lines are studied. Cells grown in this two dimensional model have flat surface and squamous morphology unlike the original cells in endometrium. A simpler approach such as growing the cells in the matrigel retains the cell morphology as seen in *in vivo* endometrium (245). With the recent advances in gene silencing technology, the monolayers provide a suitable platform for testing the individual functions and effects of the gene. Over the decades, monolayer co-cultures have yielded valuable information on receptivity, implantations and interactions between the embryos/trophoblast with the endometrial cells and have provided unprecedented opportunities to test the hypotheses by manipulating the cells easily *in vitro*. Advantages of monolayers are that it can be easily executed, manipulated and allows study of individual compartments/cells. Limitations of monolayer co-cultures are that it does not mirror the *in vivo* 3d morphology, relevant biological properties are lost due to lack of intercellular communications and interactions with extracellular matrix, which makes the translation of evidence from monolayer cultures to *in vivo* difficult.

In the field of reproductive medicine, *in vitro* 3D cell co-culture models of endometrium mimics close to *in vivo* endometrium in terms of anatomy and functional aspects and interaction of embryo with the endometrium close to nature (66, 246). In this 3D endometrial model endometrial cells (epithelial and stromal cells) are isolated and grown in layers mimicking the organization of *in vivo* endometrium and the embryo interactions can be studied in this model similar to *in vivo* conditions. Few limitations as seen with other cell culture models are also present in this model such as lack of immune cells that play a pivotal role in pregnancy and inability to study trophoblast invasion mechanism etc. and further research into this model can fine tune and overcome these limitations.

2. Aims of the thesis

The overall aim of this thesis is to expand the understanding of various factors that are affecting the endometrial receptivity and the human implantation process, thus helping in the treatment of infertility and in regulation of the fertility. The specific aims of the thesis are as follows:

1. To explore the role of Leukemia Inhibitory Factor in human embryo implantation and its viability by inhibiting the action of LIF using Poly-ethylene glycated leukemia inhibitory factor antagonist in an *in vitro* endometrial 3D cell co-culture model.

2. To explore the actions of ulipristal acetate, in the dosage used for emergency contraception, on endometrial receptivity and human embryo implantation process in an *in vitro* endometrial 3D cell co-culture model.

3. To explore the effects of two different low doses of mifepristone (0.5 μM and 0.05 μM) during the receptive phase of endometrium on endometrial receptivity and human embryo implantation process in an *in vitro* endometrial 3D cell co-culture model.

4. To identify the differential transcriptomic signatures regulated by progesterone in the epithelial and stromal compartment of the receptive endometrium of proven fertile woman using laser capture micro dissection.

3. Materials & Methods

Detailed description for materials and methods for each study is given in the corresponding original articles (Paper 1- Paper IV). However, an overview of materials and methods of all the studies will be provided here in the following sections.

3.1 Materials

3.1.1 Ethical permits

The Regional ethical review board, Stockholm, approved all the studies. We used cryopreserved supernumerary blastocysts obtained from the IVF clinic (Fertilitetscentrum, Stockholm, Sweden). As per the local regulations and existing laws, only embryos cryopreserved for more than five years, which are otherwise to be discarded or patients who are no longer interested in the embryo cryopreservation after conceiving and were willing to donate for research purpose were used in these studies. Written informed consent was obtained from all the couples and subjects.

3.1.2 Subjects

In all the studies, healthy proven fertile woman (should have had at least one spontaneous conception), aged 22-40 years with regular menstrual cycles volunteered to participate in the studies. Main inclusion criteria for the recruitment was that, none of the subjects have not used any hormonal therapy or intrauterine devices (IUDs) for contraception for three months prior to the study and were free from any gynecological pathologies. Barrier contraception was recommended, if at all the subjects were not sterilized.

3.1.3 Endometrial biopsies

The endometrial biopsies were obtained from the upper part of uterine fundus using pipelle aspirator (Prodimed, France). All subjects self examined for LH peak from cycle day 10 to LH+2 twice daily in the urine sample using LH self test urine strips (Clearplan, Searle Unipath Ltd., Bedford, UK). In paper 1, timed endometrial biopsies from fertile donors were obtained at LH+4 (n = 20), in paper 2&3 twenty biopsies on LH+4 were utilized. In paper 4, nine endometrial biopsies were obtained on day LH+2 and LH+6/7 for transcriptomics study using microarray. 27 endometrial biopsies, nine biopsies each during proliferative, mid and late secretory phase for immunohistochemical study and 30 biopsies on LH+4 day is collected for *in vitro* 3D cell culture study. In addition for determining the protein expression of ENPP3, 6 biopsies were obtained from control and treatment cycle (200mgms of mifepristone on LH+2) on day LH+6 to LH+9.

3.1.4 Uterine fluid

Uterine fluid was collected from the fertile woman with or without treatment (single dose of 200mg mifepristone on LH+2) and uterine fluid was collected on day LH+7 (Study 4). Another set of uterine fluid samples were collected for proteomic analysis during the early secretory phase LH+2 and mid secretory phase LH+8. In either sets, 2ml of water for injection grade (for Western blot analysis in paper 4) or PBS (for mass spectrometry, in paper 4) was used to collect the uterine fluid along with the uterine lavage from the uterine cavity using a modified feeding catheter (Nutrisafe 2, Fr-L.75cm, France). Of the 2ml injected, around 1ml was sucked back with the help of syringe and the fluid was centrifuged at 200g for 10min to remove contaminating blood cells and cell debris.

3.1.5 Embryos/Blastocysts

Supernumerary embryos/ blastocysts of clinically usable quality with minimum grade 3BB as per Gardner and Schoolcraft classification were used in all the studies. A total of 69 blastocysts were collected, 51 embryos survived the thawing and were used in studies as mentioned in Paper 1. In paper 2 a total of 20 embryos, 10 in the control group and another 10 in UPA treated group were used. In paper 3, a total of 28 embryos were used, 10 each in control, 0.05µM and 8 in 0.5µM mifepristone groups. For paper 4, a total of 18 blastocysts were utilized, of them 10 were used in control cultures and 8 were used in the treatment group.

3.1.6 *In vitro* treatment protocol

A modified alpha medium, herein termed as control medium was used for all the studies using *in vitro* 3D cell cultures involving embryos. All the control cultures had equal quantity of vehicle/solvent that was used for resuspension of drug. The following treatment protocols were employed for *in vitro* co-culture models,

Paper 1: 10 embryos were exposed to control medium with vehicle alone, 5 are exposed to control medium with vehicle plus polyethylene glycol (13µM, equimolar to PEG in PEGLA) (PEG) and 10 are subjected to exposure of 800pg/ml PEGLA, a LIF antagonist. A similar protocol was used for examining the direct effects of PEGLA on human embryos, 13 blastocysts per each group of control and PEGLA treated for AKT/Caspase-3 study.

Paper 2: The 3D cell cultures are exposed to UPA in a dose of 200ng/ml concentration that is equivalent to conventional dose of UPA used in emergency contraception (30mg orally).

Paper 3: Embryo endometrial co-cultures are exposed to 0.5µM Mifepristone (n=8) and 0.05µM Mifepristone (n=10) dosage in modified alpha medium.

Paper 4: A mifepristone dosage of 0.5μM is used for the embryo co-cultures to demonstrate the regulation of ENPP3 expression by progesterone.

3.2 Methods

3.2.1 Endometrial cell isolation

The endometrial biopsies collected in Ham F10 + 20% FBS + Penicillin/Streptomycin were processed immediately for individual endometrial cell isolation. Briefly, biopsy was minced into 1x1mm pieces with scalpel in Ham F10 medium and centrifuged, discarded the supernatant and Pancreatin-Trypsin Solution (0.5gms/10ml trypsin) was added and incubated at 4^0C on ice with gentle agitation, every five minutes, followed by incubation at room temperature (RT) for 10min and centrifuged. The supernatant was discarded and incubated with 5 ml FBS to prevent the digestion by remaining trypsin for 10 min and centrifuged. The supernatant is discarded and incubated with 5ml of warm collagenase type 4 solution plus DNAse mixture and incubated for 30 min at 37^0C and centrifuged and supernatant was discarded. The cell mixture was sieved in Ham F10 through a 40μ cell strainer, the epithelial glands were retained in the strainer and stromal cells were collected from the filtrate. The epithelial glands were washed, collected and incubated with collagenase type 3 solution with DNAse mixture in PBS for 30 min at 37^0C and centrifuged and resuspended in fresh Ham F10 media. The mixture was filtered through a 40μ cell strainer and epithelial cells were collected from the filtrate. Stromal and epithelial cells were resuspended in freezing mixture and the cryovials were stored in Mr. Frosty cryobox, stored at -80^0C for 24 hours and then shifted to liquid nitrogen and stored till further usage.

3.2.2 3Dimensional Endometrial Cell Co-Cultures

Endometrial 3D cell cultures were constructed with minor modifications as described earlier (66, 247). Briefly, the collagen gel type 1 (Purecol 3mg/ml, Advanced Biomatrix, USA) was mixed with 10X PBS and pH adjusted to 7.4 with NaOH and 175μl of this gel was pipetted into the Millipore cell culture insert, the gel was polymerized by incubating at 37^0C for 30 min. Stromal cells (2 million/ml) were thawed and mixed with 200μl of the gel, overlaid on the solidified collagen gel in the insert and incubated at 37^0C for 30min. After the stromal gel solidifies, a layer of thin basement membrane extract (100μg/ml) (Matrigel, Belgium) is coated on the top of the stromal gel. Epithelial cells (0.2million/ml) were thawed and mixed with modified alpha medium and pipetted onto the matrigel coating and incubated for five days at 37^0C in 5% CO_2 incubator, medium change is given for every 2 days and once the epithelial cells reach the confluency the desired treatment is started and co cultured with blastocysts for five days.

3.2.3 Immunohistochemistry (IHC)

All IHC kits were obtained from Biocare Medical, Concord, CA. The paraffin sections were deparaffinized and antigen retrieval was done in DIVA decloaker solution in 2100-retriever autoclave followed by washed in hot rinse solution. Endogenous peroxidase activity was inhibited by treating with undiluted peroxidazed for 5 min in darkness and treated with background sniper for 10 min to reduce the non-specific background during the staining. Following, the sections were incubated with the primary antibodies for an hour in RT or 4^0C overnight. Then sections were washed with TBS and incubated with MACH3 Probe (Mouse/Rabbit) corresponding to the species of primary antibody used. Afterwards, the sections were washed with TBS several times and incubated with MACH3 HRP polymer followed by incubation with betazoid DAB chromogen kit and counterstained with hematoxylin solution for a minute. The slides were dehydrated using series of increasing concentration of ethanol, washed in xylene and mounted using Pertex. The identification of the slides were blinded and semi-quantitative analysis was performed using IRS scoring method (248), separately for the glandular and stromal compartments, by two independent observers. The scoring was performed as follows: IRS (Immuno Reactive Score) = SI (staining intensity) X PP (percentage of positive stained cells) If there was a disparity of more than 25% of the score, a third independent observer analyzed the slides and the average score of the two closest results was taken for further analysis.

3.2.4 Immunofluorescence and Immunocytochemistry (Paper 1)

Immunofluorescence and immunocytochemistry were employed in the paper 1. Immunofluorescence was performed for visualizing the expression of cell survival factor Phospho AKT in the blastocysts treated with PEGLA and immunocytochemistry was performed on the embryos treated with PEGLA or controls for the apoptosis marker cleaved caspase 3. In briefly, the fixed blastocysts were permeabilized for 30 min in 0.25% Triton-X 100 and incubated in 0.1% bovine serum albumin (BSA) blocking solution. The sections were incubated with anti-cleaved caspase 3 antibody in immunocytochemistry and with anti-phospho-AKT antibody in immunofluorescence for overnight. After incubation, the sections are washed thrice in blocking solution comprising of 50mg BSA, 5 µl Tween 20 in 50ml PBS. Secondary antibody (Alexa flour 488) incubation is ensued washing step for one hour at room temperature in darkness followed by washing thrice in blocking solution and mounting of embryos is done in vecta shield and observed under Ziess Axioskop microscope. For each experiment negative controls are also performed substituting the primary antibody with isotype matched IgG with same concentration as used for primary antibody.

3.2.5 Laser capture micro dissection (LCMD) (Paper 4)

For LCMD, sections of 10 μm were prepared from the frozen endometrial biopsies using a cryotome and the sections were fixed on UV treated membrane slides using 70% ethanol at -20^{0}C for 30min. The sections were stained with Histogene^{TM} staining solution (Applied Biosystems) and around 200 epithelial and stromal cells were dissected out using PALM laser-Microbeam system (355-nm diode laser) with the assistance of PALM Robosoftware (Carl Zeiss, Germany) as per the manufacturer protocol. All steps were performed strictly in RNAse free environment, in addition a separate set of LCMD was performed and RNA extracted for real time PCR analysis apart for microarray analysis.

3.2.6 Microarray and microarray analysis (Paper 4)

Microarray for LCMD epithelial and stromal cells was performed at core facility (Bioinformatics and expression analysis in Karolinska Institutet). NUGEN Ovation Pico WTA system (Nugen Technologies) was used for RNA amplification. The amplified RNA was used to generate sense target of cDNA using NUGEN WT-Ovation Exon module version 1.0. NUGEN encore biotin module was used for the purpose of fragmentation, biotin labeling and ST cDNA preparation and microarray is performed on Affymetrix GeneChip platform (Human gene 1.0 ST array) that corresponds to 28,829 annotated genes.

The signals from CEL files of microarray were converted using Affymetrix expression console and the following particulars were used: PLIER (Probe Logarithmic Intensity Error Estimation) for summarization algorithm with PM-GCBG background correction and quantile sketch method for normalization procedure. The data was thoroughly checked for quality control and interfering outliers using R Software and differential expression analysis was performed using SAM (Significance Analysis of Microarrays) and MeV software (part of TM4 microarray suite). The differentially expressed genes obtained from the microarray analysis were further analyzed for functional and canonical pathways using IPA (Ingenuity Pathway Analysis) software (Qiagen).

3.2.7 RNA extraction and real time PCR

In paper1 RNA extraction was done from the paraffin sections of endometrial constructs and from the blastocysts by using the Picopure RNA isolation kit (Arcturus, USA) as per manufacturers protocol. In paper 2 & 3 the endometrial constructs after termination were lysed in 1ml of Trizol reagent followed by extraction of RNA, similarly in paper 4 the laser capture micro dissected endometrial epithelial and stromal cells were lysed in lysis buffer and extraction of RNA was done using picopure RNA isolation kit (Arcturus, USA). All samples were DNAse treated to remove contaminating genomic DNA and the RNA quality control was assessed on Biorad Experion Highsens automated electrophoresis system

or on Agilent 2100 bioanalyzer. Complimentary DNA (cDNA) synthesis is done from the extracted RNA by VILO superscript kit (Invitrogen) as per the manufacturers protocols. The cDNA obtained was used for RT-PCR using Step One Plus machine (Applied Biosystems) with VIC/FAM as reporting dyes. ΔΔCt comparative method was used for analyzing the RTPCR results. The list of primers and genes used for RTPCR are mentioned in the original articles.

3.2.8 Western Blot (Paper 4)

ENPP3 protein expression in the tissues and uterine fluid as mentioned in the paper 4 were analyzed by a completely automated western blot system (Protein Simple, USA) and analysis was done with its associated Compass software as per the manufacturers instructions. Initial protein quantification was done using standard BCA method and 2µg of protein either from the uterine fluid samples or from tissue lysates was used in the assay. An incubation of 45 min for primary antibody and 60 min for secondary antibody was performed and streptavidin and luminal peroxide fluorescence detection method was used. Anti-ENPP3 antibody (Sigma Lifesciences, HPA043772) is used in a 1:50 dilution.

3.2.9 Deglycosylation (Paper 4)

In order to confirm the expression of ENPP3 in glycosylated form, the uterine fluid samples and endometrial tissue lysates were treated with 1 µl/sample of PNGase-F, an enzyme that digest the glycosylated moiety, the efficiency of PNGase-F or Deglycosylation is assessed by automated western blot as mentioned under western blot.

3.2.10 Nano-ESI-LC/MS/MS (Paper 4)

The mass spectrometry (MS) for proteomic analysis in the uterine fluids was performed using nano-ESI-LC/MS/MS (Thermo fisher scientific) at University of Tartu, Estonia. SDS-PAGE was used to separate the uterine fluid into six fractions based on the molecular weight. After fractionation the proteins were reduced and alkylated and in-gel digested using trypsin and analyzed by MS using electro spray ionization technique for 2hours phased gradient method. The MS data was quantified with MaxQuant software (UniprotKB reference human proteome database) and MaxLFQ algorithm is used for the normalization of label free data.

3.2.11 Statistical Analysis

Non-parametric Mann-Whitney test was performed for analyzing immunohistochemistry data elsewhere in this thesis. Fisher's exact test is used to calculate the statistical significance for embryo implantation rates in the 3D cell culture models in paper 2, 3 and 4 whereas Chi-square test is employed for paper 1 for embryo attachment rate. Paired T Test and SAM methods were used for analyzing the microarray data and paired or unpaired t test is used for analysis of mass spectrometry data in paper 4. Kruskal Wallis test was performed for the statistics of age of patient, embryo vitrification day and blastocyst grading. For RTPCR data, based on the assumptions of normality and variance either parametric T-Test or non-parametric Mann-Whitney test is employed. All statistical tests were performed using Statistica 10 (Statsoft Inc.) and XLStat 2015 (Addinsoft) and a value <0.05 is considered statistically significant.

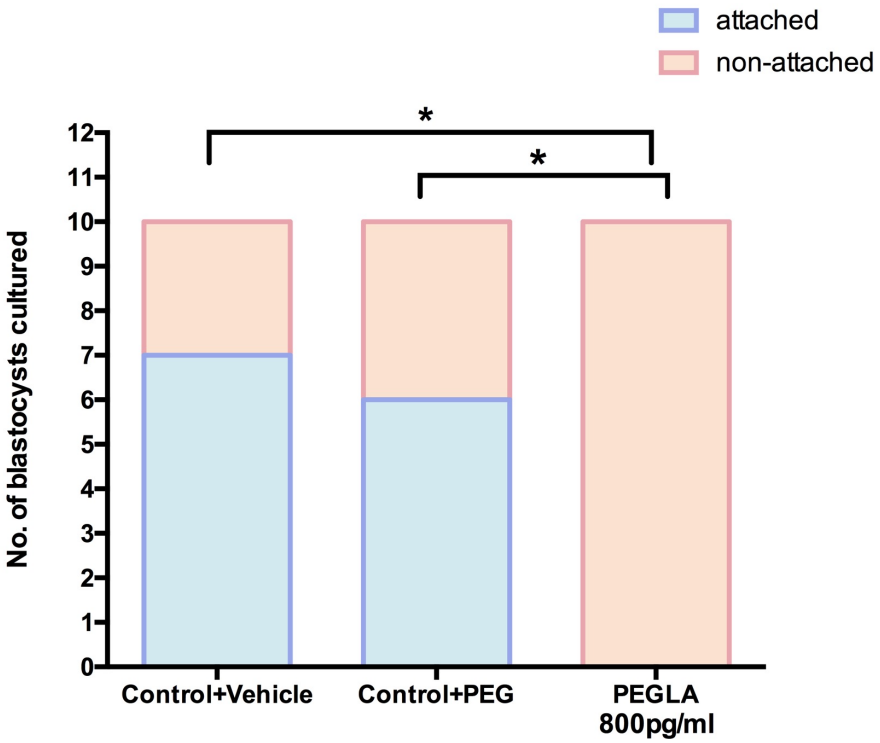
4. Results & Discussion

4.1 Paper 1: In this paper we demonstrated the role of LIF in human embryo implantation using *in vitro* 3D endometrial cell culture, the role of LIF in blastocyst viability by inhibiting the action of LIF using LIF antagonist PEGLA.

4.1.1 Results

None of the 10 embryos in PEGLA treated group were attached to the endometrial constructs, whereas 7 and 6 were attached in the control + vehicle and control + PEG groups respectively with statistical significance of $p < 0.01$ (chi-square test). No statistical significance is observed between the two control groups ($p = 0.639$). No statistical significance is seen between the age of patients ($p = 0.148$), embryo freezing day ($p > 0.05$) and blastocyst grade ($p = 0.965$) used in the study.

Figure 3: Effect of PEGLA and PEG alone on blastocyst attachment



Immunohistochemical analysis demonstrated a reduced expression of LIF and gp130 in epithelial cells of the PEGLA treated group in comparison to controls ($p=<0.05$). A similar observation was seen for LIF in RTPCR with 4.3 fold down regulation in PEGLA group ($p = 0.03$). However, gp130 expression by IHC in the stromal cells of PEGLA group

showed no significant difference from the control group. LIFR expression levels did not vary in either of the groups in stromal and epithelial cells. RTPCR analysis revealed no statistically significant differences in LIFR and gp130 expression between the control and PEGLA treated group.

In vitro, direct culture of embryos with PEGLA led to significant reduction in number of embryos hatched and blastocyst expansions ($p < 0.01$), undetectable levels of cell survival factor phopsho-AKT1 and an increased activity of apoptosis marker cleaved caspase 3 by immunofluorescence.

4.1.2 Discussion

To our knowledge, for first time we demonstrated the role of LIF in human embryo implantation by 3D endometrial co-culture model that mimics the *in vivo* endometrium to the closest. Inhibition of LIF by stable LIF inhibitor, PEGLA, inhibits embryo attachment to the cultures clarifying the role of LIF in human embryo implantation, *in vitro*. In addition, in this study we have demonstrated that LIF is required in optimal levels probably <10pg/ml in the endometrium of receptive stage for a successful embryo implantation.

It is well known that LIF expression is seen in the trophectoderm cells of the embryo and LIFR expression throughout the inner cell mass of the blastocyst suggesting an important role of LIF in development of blastocyst. This led us to study the direct role of LIF in the blastocyst development and here, we elucidated that LIF is essential for blastocyst development, expansion and hatching as PEGLA treatment abolished all the mentioned processes mediated by LIF. Furthermore, we also proved that LIF is critical for embryo survival by maintaining a balance between apoptosis factors and cell survival factors, as inhibition of LIF increased the activity of apoptosis marker cleaved caspase 3 and a down regulation of cell survival factor phospho-AKT 1.

A common approach to study the role of cytokines in clinical use is by using stable and potent inhibitor with a longer half-life and efficacy (249), therefore we used LIF inhibitor conjugated to PEG which has increased half-life and efficacy as it is already demonstrated that non-PEGylated LIF antagonist has a short serum half life of around 10-30min (250). Moreover, *in vivo* PEGLA administration has been shown to affect endometrial receptivity and decrease implantation when administered vaginally. In order to rule out the adverse effects of PEG, if any, we demonstrated, in another control group treated with PEG, had no difference in the blastocyst attachment rate compared with controls with vehicle. Similarly, *in vivo* PEG administration in mice models had no effect on the implantation (251).

Experimental evidences indicate that PEGLA blocks LIF activated STAT3 phosphorylation in the endometrial epithelium during the pre implantation period (252). A similar observation is seen in our study – a reduced expression of LIF and its signal

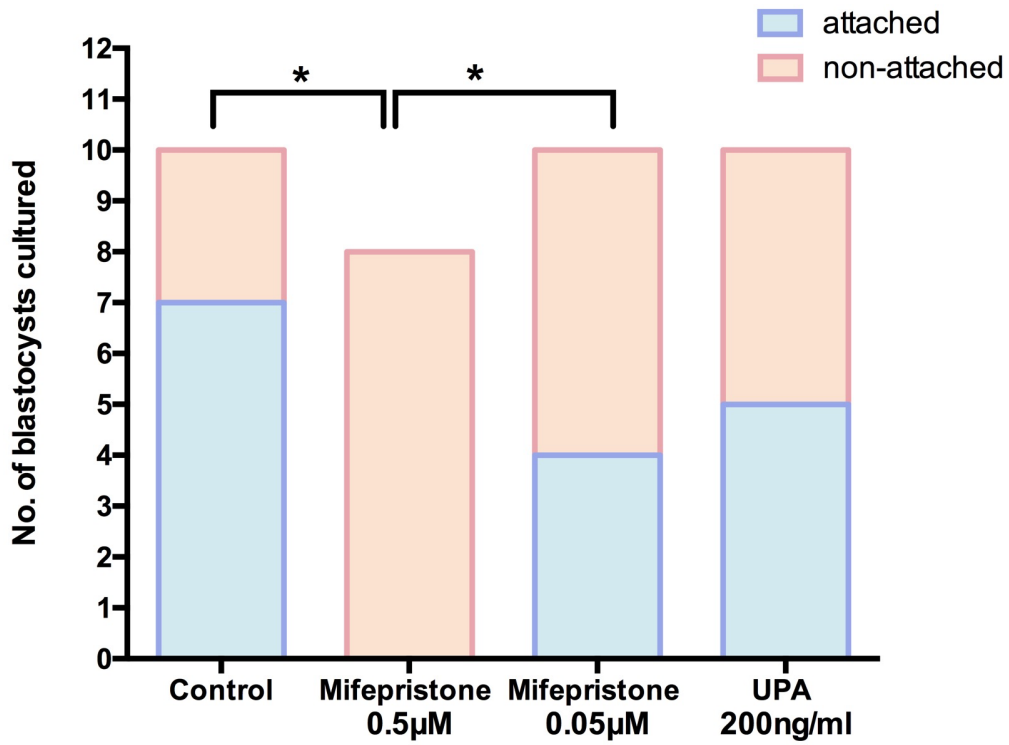
transductor gp130 in the epithelial cells as visualized by IHC and RTPCR analysis for LIF confirming the actions of PEGLA on LIF signal transduction at endometrial level. Earlier studies conducted in different animals species have tried to explore the possibility of developing non-hormonal contraceptives targeting LIF (253), and the results from this study indicate a strong possibility for the development of non-hormonal contraceptives targeting endometrial LIF.

4.2 Paper 2: In this study we demonstrated the effect of UPA used for emergency contraception (30mg) on endometrial receptivity and embryo attachment in a 3D endometrial co-culture model.

4.2.1 Results

In the UPA treatment (200ng/ml) group 50% of the blastocysts attached to the constructs whereas in the control group 70% attached to the constructs (Figure 2). No statistically significant difference is seen between the groups in blastocyst attachment rate (fishers exact test, p=0.650).

Figure 4: Blastocyst attachment rate in control and treatment groups



*** p value < 0.05**

In order to observe the effects of UPA on embryo implantation and endometrial receptivity, seventeen related genes were analyzed from the constructs with RTPCR (Table 2). The genes studied include cytokines, cell adhesion molecules, transcription factors, growth factors, decidualization markers etc. Of the 17 genes analyzed by RTPCR, six of them, namely HAND2, OPN, HBEGF, CALCR, FGF2 and IL6, were differentially expressed between the groups and were statistically significant with the treatment. HAND2, OPN, FGF2 and CALCR were significantly downregulated, whereas HBEGF and IL6 were significantly upregulated in the treatment group. Progesterone receptor (PGR) expression was seen in all the samples irrespective of treatment, but significant down regulation of PGR was seen in UPA treated group ($p<0.001$). No statistical significance in PGR expression was seen between the embryo attached and non-attached groups in the treatment group.

Table 2: Gene expression levels of selected endometrial receptivity markers, embryo implantation process and decidualization analyzed by RTPCR after exposure to UPA 200ng/ml (study 2) and two low doses of mifepristone (0.5µM and 0.05µM) (study 3).

*** p value < 0.05**

Gene	UPA 200ng/ml Fold change	UPA 200ng/ml p-value	Mifepristone 0.5µM Fold change	Mifepristone 0.5µM p-value	Mifepristone 0.05µM Fold change	Mifepristone 0.05µM p-value
Transcription factors						
HAND2	-2.74	0.003*	-2.28	0.0040*	-2.32	0.004*
HOXA10	-1.07	0.307	-7.94	0.0003*	-7.41	0.003*
FOXO-1	-2.01	0.055	-4.54	0.0001*	-7.56	0.0001*
COUP-TF2	-2.29	0.306	7.88	0.0001*	6.75	0.008*
Cytokines						
LIF	-1.39	0.107	-2.15	0.005*	-2.13	0.005*
IL-6	2.63	0.025*	1.19	0.165	3.51	0.003*
IL1A	1.26	0.065	-1.15	0.472	-1.56	0.028*
Growth factors						
FGF2	-2.06	0.023*	-12.04	0.0001*	-13.21	0.0001*
CSF-1	-1.09	0.48	1.36	0.088	1.06	0.999
HBEGF	2.48	0.009*	1.03	0.670	0.6	0.154
VEGFA	1.09	0.596	-1.29	0.219	-3.77	0.0001*
Decidualization factors						
PRL	-1.47	0.427	-8.79	0.0001*	-18.68	0.0001*
IGFBP1	-1.34	0.653	-2.29	0.0002*	-2.38	0.003*
Receptors						
OPN	-3.89	0.004*	-2.96	0.005*	-10.12	0.0001*
PGR	-4.89	0.001*	-2.14	0.001*	-1.85	0.01*
MUC1	-1.44	0.391	1.7	0.154	1.43	0.428
CALCR	-2.52	0.016*	-	-	-	-

4.2.2 Discussion

UPA is a second generation SPRM with both agonistic and antagonistic actions on PRs. UPA in a single dose of 30 mg is used as emergency contraception, however the exact mechanism of action of UPA on the receptive endometrium and implantation is not clearly known. In this study, we demonstrated for the first time the action of UPA used as emergency contraceptive, on blastocyst attachment rate and effects on various endometrial receptivity markers *in vitro* using a 3D endometrial model. This dose of UPA did not affect the blastocyst attachment significantly in comparison to the control group and most of the receptivity markers were unaffected, as demonstrated in this study.

Technically and ethically it is impossible to study the effects of SPRMs or any anti progestins *in vivo* and most of the available data of UPA on implantations has been obtained from animal models and non-human primates, which have contributed valuable information in understanding the mechanism of anti progestins (254, 255). The 3D cell culture model that we employed here has been proven to be useful in understanding the mechanism of actions of various antiprogestins, cytokines on implantation process (113, 256, 257) and is the closest available *in vitro* model of endometrium. The mean serum concentration of UPA observed after a dosage of 30 mg orally is 176 ± 89ng/ml (258) and in this study we used a continuous dosage of 200 ng/ml during the culture that is slightly more than mean serum concentration. However it has to be noted that steroid hormone concentrations are seen in higher levels in the endometrial tissue rather than the serum, though we do not know *in vivo* levels of UPA at endometrial level when used at the above dose.

A quick search on endometrial receptivity and implantation markers would yield numerous biomarkers such as cytokines, CAMS, transcription factors and so on, and with the advancing technologies this number is increasing exponentially but none of them has been proved to be the best receptivity marker. Previous studies conducted to explore the efficacy of UPA in endometrial receptivity were investigated for a limited number of markers (259), hence, in this study we explored the effect of UPA on a large number of receptivity markers belonging to different classes and selected 17 important markers that are well reviewed in the literature including the decidualization markers.

In the current study we found that most of the genes that are important and vital for implantation such as cytokines LIF, IL1A, growth factors CSF-1, transcription factors COUP-TF2, FOXO1, HOXA10, enzyme SGK1, angiogenesis factor VEGFA and MUC1 are unaltered in the endometrial constructs exposed to UPA. Several studies have demonstrated the importance of LIF and our study using 3D culture model showed that inhibiting LIF in cultures prevented implantation and embryo viability (257), as was also with FOXO1, HOXA-10 and COUP-TF2. In the present study it is evident that UPA did not have any significant effect on the above-mentioned factors despite the pharmacological dose used in

the culture. Decidualization is an important physiological phenomenon triggered by the implantation event and Prolactin and IGFBP1 are considered important biomarkers of decidualization and lack of decidualization has been linked to implantation and pregnancy failure (260). In the event of implantation, expression levels of PRL and IGFBP1 are increased by many fold, in contrast we observed no significant changes in the expression levels of this markers with UPA treatment *in vitro*. This could be due to failure of trophoblast cells to invade the 3D constructs unlike in the *in vivo* conditions, a limitation of this 3D model in the study of invasion mechanism. Further fine-tuning of this model may overcome this limitation.

In this study we found six genes, IL6, HBEGF, HAND2, FGF2, OPN and CALCR that are differentially regulated with UPA exposure. IL6 and HBEGF were significantly upregulated and the rest four were downregulated. Reports indicate that with the trophoblast conditioned medium an increase in IL6 expression (261) is seen, the expression of IL6 is in line with the mentioned reports. HAND2 exerts its action of anti proliferation on epithelial cells through the suppression of FGF2 signalling (186). Increased expression of HAND2, FGF2, HBEGF, OPN and CALCR are seen in the preimplantation phase endometrium. This increased expression levels observed with the UPA treatment could be attributed to the agonist action of UPA towards the progesterone receptors. Moreover, receptivity is regulated by a cascade of mechanisms of several factors but not by a single marker. However, UPA is effective in preventing pregnancies if administered before the ovulation by delaying follicular maturation.

Logically and arguably, implantation itself could be the best marker for the receptiveness of endometrium. Therefore, from the findings we presume that UPA does not affect the receptivity of endometrium and ultimately implantation, as was evident in this study.

4.3 Paper 3: In this study, we explored the effects of two low doses of mifepristone on endometrial receptivity and blastocyst attachment using an *in vitro* 3D endometrial co-culture model.

4.3.1 Results

In control group, 70% blastocysts were attached to the endometrial constructs, in 0.05μM group, 40% were attached whereas in the 0.5μM group none of the blastocysts attached to the constructs (fishers exact p= 0.004) (Figure 2). However, no statistical significance was seen between the control and 0.05μM mifepristone group with the embryo attachment rate (fishers exact p=0.369).

To explore the effect of two low doses of mifepristone on endometrial receptivity and implantation, 16 genes that are considered to be biomarkers of endometrial receptivity and implantation were analyzed in the endometrial constructs by RTPCR (Table 2). In both the treatment groups PGR expression was measured and was found to be significantly downregulated by 2.14 fold (p = 0.001) in 0.05μM and 1.85 folds (p=0.01) in 0.5μM mifepristone group. Of the tested 16 markers by RTPCR nine of them were differentially expressed in both the treatment groups, whereas IL6, IL1A and VEGFA were differentially expressed in only 0.5μM mifepristone treated group in comparison to control group.

4.3.2 Discussion

We have previously demonstrated the effect of 10μM mifepristone on endometrial receptivity and embryo attachment (113) and in this study we explored the effects of two low doses of mifepristone on endometrial receptivity and subsequent blastocyst implantation in a well-established and proven endometrial 3D cell co-culture model. For the first time, we show that exposure of receptive endometrium to a 0.5μM dose of mifepristone alters the molecular signature of the receptive endometrium and inhibits implantation.

In vivo, the endometrium is receptive during the WOI that spans between LH+7 to LH+11 under the influence of progesterone hormone. In order to mimic this situation *in vitro*, the stromal and epithelial cells in the endometrial construct were primed with progesterone to simulate *in vivo* condition as closely as possibly we can. Moreover, we have shown that the *in vitro* model that is employed is suitable for studying the initial phase of the implantation i.e. adhesion phase.

Mifepristone in the oral dose of 5mg has been shown to inhibit ovulation and a daily administration of 5 mg mifepristone in treatment of leiomyoma causes marked endometrial changes (214, 215, 262). Mifepristone dosage of 0.5 μM used in the current *in vitro* study represents the *in vivo* concentration attained in the uterine tissue after a single 5 mg oral dose.

Interestingly, in a clinical trial of mifepristone (5mg/week) only as the contraceptive agent, a reduced fertility was observed, but did not completely prevent the pregnancy (263). Here we wanted to identify a low dose of mifepristone that could inhibit implantation. Since pronounced changes were observed at the molecular and functional levels with 0.5μM treatment for 5 days, we reduced the dosage to one tenth of 0.5μM i.e. 0.05μM. An ideal study to derive the dose response effect of mifepristone would be to use graded concentrations with a series of experiments. However due to limitation of availability of blastocysts and endometrial biopsies we tested only two low doses of mifepristone.

Of all the receptivity markers analyzed from the endometrial constructs no statistically significant difference was observed between 0.5μM and 0.05μM treatment groups, except for IL6. We hypothesize that this behavior of IL6 as, an inflammatory trigger associated with the cells to repair the response due to the withdrawal of progesterone action by a low dose of 0.05μM mifepristone, since no change in the embryo attachment rate was seen in comparison to control group. Moreover, it has been shown that mifepristone per se has no adverse effects on the embryo in a primate model (264), therefore we conclude that findings of the present study are not the direct effect of mifepristone on blastocyst but are due to the effects on endometrium.

4.4. Paper 4: In this study we explored the gene expression changes in the stromal and epithelial compartment regulated by progesterone in receptive endometrium using laser capture micro dissection. We propose that glycosylated ENPP3/cd203c, a progesterone-regulated factor, has the potential to be used as a non-invasive test for diagnosing endometrial receptivity.

4.4.1 Results

In this study, LCMD of epithelial and stromal cells followed by RNA extraction yielded a minimum of 500pg RNA. Microarray data analysis revealed 47 genes differentially regulated with antiprogestin mifepristone treatment, 32 up and 15 down regulated, in the epithelial compartment. In the stromal compartments 85 genes were differentially expressed, 79 of them were up regulated and 6 genes were down regulated with antiprogestin mifepristone treatment. The 132 differentially expressed genes in both the stromal and epithelial compartment were analyzed for canonical pathways and biological networks using bioinformatics tool IPA. The dataset was analyzed for the upstream regulators that are responsible for the gene expression changes in the given experimental dataset and it was revealed that CBX5 (Chromebox Homolog 5), a transcription factor, was inhibited whereas CSF2 (Colony stimulating Factor 2) and EBF1 (early B cell Factor 1) were activated, based on the Z-scores calculated by the IPA.

These microarray findings were revalidated by RTPCR and IHC analysis for selected genes based on the importance and availability of the antibodies. RTPCR was done for 13 selected genes in stromal compartment and 11 in glandular compartment and is in line with the microarray findings (Table 3). We found a gene of interest, ENPP3 that is downregulated by 55-fold in the stromal compartment and 9-fold in epithelial compartment by RTPCR analysis. 7 genes were selected to study by IHC and found that except ENPP3, none of them were significantly regulated between the groups. Immunostaining for ENPP3 was seen in all the samples of control group and statistical analysis demonstrated a significant down regulation in the treatment group (p=0.0007).

Table 3: Validation of microarray results of selected genes that were differentially regulated with P inhibition by RTPCR both in the endometrial stromal and glandular compartments

Stromal Compartment			Glandular Compartment		
Gene	**Fold Change**	**P Value**	**Gene**	**Fold Change**	**P Value**
MT1G	-135.552	0.0082*	**MT1G**	-519.598	0.0053*
ENPP3	-55.928	0.0348*	**ENPP3**	-9.465	0.0155*
MT2A	-3.514	0.0307*	**MT2A**	-7.016	0.0028*
SFRP4	8.769	0.0128*	**UBE2E2**	7.052	0.0237*
CPM	29.055	0.0051*	**SFRP4**	16.251	0.0001*
SOD2	-1.472	0.3399	**RPL27A**	4.262	0.0955
BCL11A	-1.212	0.3785	**BCL11A**	8.302	0.1318
ATP6V0E1	1.277	0.2270	**POSTN**	2.686	0.3518
HMGN5	-1.092	0.8519	**STC1**	-5.498	0.4030
SNORA3	-1.268	0.8885	**RHOJ**	1.480	0.6211
RPL27A	1.127	0.9000	**VPS53**	1.073	0.9423
SMARCA1	1.593	0.9581			
CTSC	1.993	0.1981			

*** p value < 0.05**

We investigated the expression of ENPP3 in the endometrium and found it to be significantly down regulated by 59-fold in the epithelial compartment in microarray (p=0.04) in the mifepristone treated group. ENPP3 expression as analyzed by IHC, was localized at the apical surface of the epithelium in the luminal and glandular epithelium, very scanty expression was seen in the glands of treatment group. In contrast, ENPP3 expression was not seen in the stromal compartment either in the receptive or non-receptive endometrium by

immunohistochemical analysis and western blot, although its expression was seen at mRNA level in the stromal compartment. Further, we discovered that ENPP3 shows a cyclical expression in menstrual cycle, being highest during the WOI and least in the proliferative phase, an expression similar to progesterone secretion. Statistical significance was seen in the expression of ENPP3 in mid secretory phase compared to proliferative phase ($p=0.0001$), however, no significant difference is seen between mid secretory and late secretory phase as analyzed by IHC technique.

Furthermore, we investigated the ENPP3 expression in the uterine lavages and endometrial tissue lysates by completely automated western blot. A strong immunoband was observed at 165kD rather than at 100kD both in the uterine lavages and endometrial tissue lysates indicating glycosylated form of ENPP3. A significant down regulation was seen in the treatment group of both uterine fluid ($p=0.002$) and tissue lysates ($p=0.002$), however, abundant expression of ENPP3 is found in the tissue lysates compared to the uterine lavages. Thereafter, we have evaluated and confirmed that ENPP3 exists in the glycosylated form by deglycosylation using PNGase-F enzyme, which cleaves the glycoaminidase link and were evaluated again by western blot. A shift in the immunoband from 165kd to 110kd was observed in the deglycosylated samples, confirming the existence of ENPP3 in glycosylated form. We also analyzed the uterine fluid from receptive and non-receptive phase for quantifying ENPP3 expression by nano-ESI-LC/MS/MS and discovered ENPP3 to be upregulated in the progesterone dominant endometrium by 35-fold with respect to non-receptive phase (LH+2) uterine fluid.

Seven of ten blastocysts attached to the endometrial constructs and the expression of ENPP3 in these constructs was significantly high compared to the mifepristone treated group suggesting a regulation of ENPP3 by progesterone.

4.4.2 Discussion

In the current study, we showed that ENPP3, a progesterone-regulated factor, is expressed in the epithelial glands and uterine secretions and exists in a glycosylated form. Also, we demonstrated the regulation of ENPP3 by progesterone in a functional study with blastocyst cultures on endometrial 3D cell cultures and treated with antiprogestin. It was found that ENPP3 expression was significantly down regulated with inhibition of blastocyst implantation in the treatment group. Since a comparable level of endometrial ENPP3 expression reflects in the uterine fluid, we propose ENPP3 as a progesterone regulated endometrial receptivity marker that has potential capability to be used in the non-invasive diagnosis of receptivity. However, this hypothesis has to be tested in a clinical setup in various fertility disorders like RIF with a large number of patients for validation.

Several studies have been done using the transcriptomics approach to identify potential biomarkers of endometrial receptivity with an aim to translate them into clinical use

(240, 265, 266). Interestingly none of these studies and studies that correlated mRNA and protein levels during the receptive phase and non-receptive phase have reported the expression of ENPP3. Here, for the first time, we report ENPP3 is expressed during the mid secretory phase/WOI during which the domination by progesterone is seen in the endometrium. In addition, we demonstrated its role in implantation by using a well-established 3D model, however it would be interesting to explore the specific mechanism of action of ENPP3 in receptivity and implantation, particularly in infertile patients.

The encouraging findings of ENPP3 in uterine fluid makes it a promising marker for the diagnosis of endometrial receptivity using uterine fluid sample that could be collected by less invasive technique. Moreover, uterine fluid sampling in a cycle where ET is planned does not affect the implantation rate or pregnancy (267), hence non-invasive measurement of ENPP3 levels in uterine fluid could be of benefit to assess the receptivity of the endometrium in the same embryo that of transfer cycle.

Majority of the studies using omics approach used the endometrial tissue as a whole, which gives a cumulative picture of all cell types present in the endometrium. In this study, rather than going with the endometrial tissue lysis directly, we used LCMD to specifically isolate the major cells of endometrium, stromal and epithelial cells and analyzed the genomic signature of these compartments individually, which gives a better representation of endometrial receptivity based on specific cell type. Using this approach we have discovered ENPP3 that is specific to epithelial compartment and validated the initial microarray findings at mRNA and protein level by RTPCR, immunohistochemistry and western blotting techniques.

One of the important limitations with human samples in research is the availability of the sample in enough quantity for analysis; normally a sufficient quantity is required for protein analysis. Since we had a limited quantity of tissue, this limitation was taken care of by utilizing a recently introduced sensitive and specific rapid method of western blot, simple Wes. Simple Wes western blot is suitable for analyzing low level of proteins in a very small sample since very small quantity of protein could be obtained from the uterine fluid from women. Also, we studied the expression of ENPP3 in both the receptive and non-receptive phase of uterine fluid by sensitive, label free protein technology mass spectrometry (nano-ESI-LC/MS/MS).

Regulation of ENPP3 in the endometrium is intriguing. Interestingly, mRNA of ENPP3 is upregulated by 55-fold change in the stromal compartment of the receptive endometrium compared to stromal cells in the non receptive phase endometrium, however there is no detectable protein either by immunohistochemistry or by western blot in stromal compartment of both the groups. This typical behavior of high mRNA and no protein not only reflects the post-translational mechanisms but also may have a biological role that needs to be addressed further.

ENPP3 expression in the endometrium shows a similar trend to progesterone, being highest in the mid secretory phase, followed by late secretory phase and least expressed in the proliferative phase suggesting a considerable role in regulation by progesterone and a role in endometrial receptivity. Here, we also have shown that ENPP3 is expressed in the glycosylated form. Glycosylation is known to play an important role in determining the protein structure, stability, function, protein-protein interactions and determines the cellular responses to the exogenous factors.

ENPP3 is a type II transmembrane protein and expression is reported in the mast cells and basophils and is considered as a marker of allergic reactions (268). It belongs to the ectoenzymes family possessing ATPase and ATP pyrophosphatase activity and is involved in hydrolysis of extracellular nucleotides. Recently, it has been shown by Korekane et al. that ENPP3 regulates the glycosyltransferase activity that facilitates glycosylation of many proteins and can modify the activity of glycans (269). Glycans are also seen in the uterine secretions and in endometrium, demonstrating an important role in endometrial receptivity. ENPP3 is a newly described molecule in endometrium; hence less is known about its role in receptivity. The only protein reported from ENPP family in endometrium is ENPP5, however its role is yet to be elucidated (270).

5. Limitations

1. The study in paper 1 is mainly focused only on a single cytokine LIF, its role in embryo implantation and endometrial receptivity, however it has to be noted that *in vivo* the implantation process and endometrial receptivity is a complex phenomenon with involvement of several cytokines, cell adhesion molecules and other factors. A combination of cytokines that are present in the uterine fluid will throw more light in understanding the roles of cytokines in human embryo implantation.

2.Though the 3D endometrial co-culture model used in all the studies is close to *in vivo* human endometrium, still an improvement in the model is needed with the addition of other cell types such as uNK, endothelial cells, macrophages and other factors that are seen in the natural endometrium.

3.Assessment of blastocyst attachment to the constructs is done by washing the cultures at the time of culture termination after 5 days; this may risk the loosely attached blastocysts to the constructs, so developing a better imaging techniques to visualize the adhesion gives more valuable information.

4. Scarcity in getting timed endometrial biopsies from fertile women without any contraception use and embryos for research purpose makes an important limitation for using small sample size in all the studies.

5. The 3D endometrial co-culture model is only limited to the attachment phase of the implantation and in depth analysis of post attachment events could give valuable information of UPA in regard to the endometrial receptivity and implantation. Hence, further modification of the model is needed to study the mechanism of implantation apart from the adhesion phase.

6. Gene expression analysis by RTPCR in papers 2-4 is a cumulative result of both the stromal and epithelial cells present in the co-culture that may not give a true picture of molecular expression from the specific cell types in the endometrium. Thus, isolating the stromal and epithelial cells separately from the constructs and analysis would be highly significant.

7. In *in vivo* conditions, the drugs are metabolized by liver and are cleared from the circulation, however this mechanism is absent in any *in vitro* model that marks an important general limitation in studying the dose responses of a given drug.

6. Conclusions

1. We have shown a critical role of LIF on the viability of the blastocyst by using LIF inhibitor, PEGLA in a well-established 3D endometrial cell co-culture model.

2. UPA dosage used in emergency contraception (30mg single dose) does not affect endometrial receptivity and embryo implantation, although few biomarkers involved in endometrial receptivity and implantation process are altered to a minor extent as studied *in vitro*.

3. Mifepristone in a dose of 0.5μM successfully inhibited implantation of the human embryos in the co-cultures by affecting endometrial receptivity; most of the studied receptivity biomarkers were significantly down regulated with 0.5μM mifepristone. However, 0.05μM mifepristone affected the endometrial receptivity with no effect on implantation of human blastocysts *in vitro*.

4. ENPP3 is proposed as a molecular marker for progesterone regulated endometrial receptivity.

7. Future directions

1. Our findings have opened new potential for using PEGLA as a non-steroidal fertility regulating agent.

2. The knowledge on the role of LIF in the embryo survival mechanisms may help infertile women with uterine LIF deficiencies in improving the pregnancy rates.

3. Addition of LIF to the culture media may help in obtaining an increased rate of healthy embryos

4. In a low dose regimen, mifepristone could be developed as an on demand contraceptive.

5. Findings with ENPP3 could be used in developing a less invasive diagnostic test for diagnosing progesterone regulated endometrial receptivity that may help in screening woman seeking IVF treatment for their endometrial receptivity.

6. Further study the functional role of ENPP3 in endometrial receptivity and embryo implantation process

7. An inhibitor or molecule that alters the function of ENPP3 could be developed and potentially used for fertility regulation.

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To living life to the fullest…….!

9. Bibliography

1. Quaas A, Dokras A. Diagnosis and treatment of unexplained infertility. Rev Obstet Gynecol. 2008;1(2):69-76.
2. Mascarenhas MN, Flaxman SR, Boerma T, Vanderpoel S, Stevens GA. National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. PLoS Med. 2012;9(12):e1001356.
3. Pandian Z, Bhattacharya S, Vale L, Templeton A. In vitro fertilisation for unexplained subfertility. Cochrane Database Syst Rev. 2005(2):CD003357.
4. Bucci M, Murphy CR. Hormonal control of enzyme activity during the plasma membrane transformation of uterine epithelial cells. Cell Biol Int. 2001;25(9):859-71.
5. McLennan CE, Rydell AH. Extent of endometrial shedding during normal menstruation. Obstetrics and gynecology. 1965;26(5):605-21.
6. Mishra B, Kizaki K, Sato T, Ito A, Hashizume K. The role of extracellular matrix metalloproteinase inducer (EMMPRIN) in the regulation of bovine endometrial cell functions. Biology of reproduction. 2012;87(6):149.
7. Lee SK, Kim CJ, Kim DJ, Kang JH. Immune cells in the female reproductive tract. Immune Netw. 2015;15(1):16-26.
8. Lee JY, Lee M, Lee SK. Role of endometrial immune cells in implantation. Clin Exp Reprod Med. 2011;38(3):119-25.
9. Wilkens J, Male V, Ghazal P, Forster T, Gibson DA, Williams AR, et al. Uterine NK cells regulate endometrial bleeding in women and are suppressed by the progesterone receptor modulator asoprisnil. J Immunol. 2013;191(5):2226-35.
10. King A. Uterine leukocytes and decidualization. Hum Reprod Update. 2000;6(1):28-36.
11. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature. 2011;473(7347):298-307.
12. Kayisli UA, Luk J, Guzeloglu-Kayisli O, Seval Y, Demir R, Arici A. Regulation of angiogenic activity of human endometrial endothelial cells in culture by ovarian steroids. The Journal of clinical endocrinology and metabolism. 2004;89(11):5794-802.
13. Helmestam M, Lindgren KE, Stavreus-Evers A, Olovsson M. Mifepristone-exposured human endometrial endothelial cells in vitro. Reprod Sci. 2014;21(3):408-14.
14. Schwab KE, Gargett CE. Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. Hum Reprod. 2007;22(11):2903-11.
15. Masuda H, Anwar SS, Buhring HJ, Rao JR, Gargett CE. A novel marker of human endometrial mesenchymal stem-like cells. Cell Transplant. 2012;21(10):2201-14.
16. Schwab KE, Hutchinson P, Gargett CE. Identification of surface markers for prospective isolation of human endometrial stromal colony-forming cells. Hum Reprod. 2008;23(4):934-43.
17. Cervello I, Mas A, Gil-Sanchis C, Peris L, Faus A, Saunders PT, et al. Reconstruction of endometrium from human endometrial side population cell lines. PLoS One. 2011;6(6):e21221.
18. Challen GA, Little MH. A side order of stem cells: the SP phenotype. Stem Cells. 2006;24(1):3-12.

19. Gil-Sanchis C, Cervello I, Khurana S, Faus A, Verfaillie C, Simon C. Contribution of different bone marrow-derived cell types in endometrial regeneration using an irradiated murine model. Fertil Steril. 2015;103(6):1596-605 e1.

20. Taylor HS. Endometrial cells derived from donor stem cells in bone marrow transplant recipients. JAMA. 2004;292(1):81-5.

21. Maruyama T. Stem Cells in the Uterus: Past, Present and Future. Semin Reprod Med. 2015;33(5):315-6.

22. Guzeloglu-Kayisli O, Basar M, Arici A. Basic aspects of implantation. Reproductive biomedicine online. 2007;15(6):728-39.

23. Tang B, Gurpide E. Direct effect of gonadotropins on decidualization of human endometrial stroma cells. J Steroid Biochem Mol Biol. 1993;47(1-6):115-21.

24. Tabanelli S, Tang B, Gurpide E. In vitro decidualization of human endometrial stromal cells. J Steroid Biochem Mol Biol. 1992;42(3-4):337-44.

25. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Am J Obstet Gynecol. 1975;122(2):262-3.

26. Johannisson E, Parker RA, Landgren BM, Diczfalusy E. Morphometric analysis of the human endometrium in relation to peripheral hormone levels. Fertil Steril. 1982;38(5):564-71.

27. Li TC, Rogers AW, Dockery P, Lenton EA, Cooke ID. A new method of histologic dating of human endometrium in the luteal phase. Fertil Steril. 1988;50(1):52-60.

28. Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. Endocrinology. 2006;147(3):1097-121.

29. Grunfeld L, Walker B, Bergh PA, Sandler B, Hofmann G, Navot D. High-resolution endovaginal ultrasonography of the endometrium: a noninvasive test for endometrial adequacy. Obstetrics and gynecology. 1991;78(2):200-4.

30. Lindhard A, Ravn V, Bentin-Ley U, Horn T, Bangsboell S, Rex S, et al. Ultrasound characteristics and histological dating of the endometrium in a natural cycle in infertile women compared with fertile controls. Fertil Steril. 2006;86(5):1344-55.

31. Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, et al. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. Hum Reprod. 2004;19(10):2319-24.

32. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. Fertil Steril. 2001;76(6):1175-80.

33. Scott R, Seli E, Miller K, Sakkas D, Scott K, Burns DH. Noninvasive metabolomic profiling of human embryo culture media using Raman spectroscopy predicts embryonic reproductive potential: a prospective blinded pilot study. Fertil Steril. 2008;90(1):77-83.

34. Gardner DKaS, W. B, editor 'Towards reproductive certainty´. In vitro culture of human blastocyst. Plenary Proceedings of the 11th World Congress on IVF and Human Reproductive Genetics; 1999; New York.

35. Bergh PA, Navot D. The impact of embryonic development and endometrial maturity on the timing of implantation. Fertil Steril. 1992;58(3):537-42.

36. Psychoyos A. Uterine receptivity for nidation. Annals of the New York Academy of Sciences. 1986;476:36-42.

37. Li TC, Lenton EA, Dockery P, Cooke ID. A comparison of some clinical and endocrinological features between cycles with normal and defective luteal phases in women with unexplained infertility. Hum Reprod. 1990;5(7):805-10.

38. Farhi J, Ben-Haroush A, Andrawus N, Pinkas H, Sapir O, Fisch B, et al. High serum oestradiol concentrations in IVF cycles increase the risk of pregnancy complications related to abnormal placentation. Reproductive biomedicine online. 2010;21(3):331-7.

39. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A. 1993;90(23):11162-6.

40. Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, et al. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A. 1998;95(26):15677-82.

41. Filardo EJ, Quinn JA, Bland KI, Frackelton AR, Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol. 2000;14(10):1649-60.

42. Yang H, Kim TH, Lee HH, Choi KC, Hong YP, Leung PC, et al. Expression of calbindin-D28k and its regulation by estrogen in the human endometrium during the menstrual cycle. Reproductive biology and endocrinology : RB&E. 2011;9:28.

43. Opperman LA, Saunders TJ, Bruns DE, Boyd JC, Mills SE, Bruns ME. Estrogen inhibits calbindin-D28k expression in mouse uterus. Endocrinology. 1992;130(3):1728-35.

44. Afshar Y, Miele L, Fazleabas AT. Notch1 is regulated by chorionic gonadotropin and progesterone in endometrial stromal cells and modulates decidualization in primates. Endocrinology. 2012;153(6):2884-96.

45. Afshar Y, Stanculescu A, Miele L, Fazleabas AT. The role of chorionic gonadotropin and Notch1 in implantation. J Assist Reprod Genet. 2007;24(7):296-302.

46. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Jr., et al. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. 1995;9(18):2266-78.

47. Lippman M, Monaco ME, Bolan G. Effects of estrone, estradiol, and estriol on hormone-responsive human breast cancer in long-term tissue culture. Cancer Res. 1977;37(6):1901-7.

48. Mote PA, Balleine RL, McGowan EM, Clarke CL. Heterogeneity of progesterone receptors A and B expression in human endometrial glands and stroma. Hum Reprod. 2000;15 Suppl 3:48-56.

49. Jacobsen BM, Horwitz KB. Progesterone receptors, their isoforms and progesterone regulated transcription. Mol Cell Endocrinol. 2012;357(1-2):18-29.

50. Giangrande PH, McDonnell DP. The A and B isoforms of the human progesterone receptor: two functionally different transcription factors encoded by a single gene. Recent Prog Horm Res. 1999;54:291-313; discussion -4.

51. Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM. Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. Science. 2000;289(5485):1751-4.

52. Gao J, Mazella J, Tang M, Tseng L. Ligand-activated progesterone receptor isoform hPR-A is a stronger transactivator than hPR-B for the expression of IGFBP-1 (insulin-like growth factor binding protein-1) in human endometrial stromal cells. Mol Endocrinol. 2000;14(12):1954-61.

53. Cole LA. Hyperglycosylated hCG, a review. Placenta. 2010;31(8):653-64.

54. Kalantaridou SN, Zoumakis E, Makrigiannakis A, Godoy H, Chrousos GP. The role of corticotropin-releasing hormone in blastocyst implantation and early fetal immunotolerance. Horm Metab Res. 2007;39(6):474-7.

55. Makrigiannakis A, BenKhalifa M, Vrekoussis T, Mahjub S, Kalantaridou SN, Gurgan T. Repeated implantation failure: a new potential treatment option. Eur J Clin Invest. 2015;45(4):380-4.

56. Zhu LJ, Cullinan-Bove K, Polihronis M, Bagchi MK, Bagchi IC. Calcitonin is a progesterone-regulated marker that forecasts the receptive state of endometrium during implantation. Endocrinology. 1998;139(9):3923-34.

57. Ding YQ, Zhu LJ, Bagchi MK, Bagchi IC. Progesterone stimulates calcitonin gene expression in the uterus during implantation. Endocrinology. 1994;135(5):2265-74.

58. Kumar S, Zhu LJ, Polihronis M, Cameron ST, Baird DT, Schatz F, et al. Progesterone induces calcitonin gene expression in human endometrium within the putative window of implantation. The Journal of clinical endocrinology and metabolism. 1998;83(12):4443-50.

59. Xiong T, Zhao Y, Hu D, Meng J, Wang R, Yang X, et al. Administration of calcitonin promotes blastocyst implantation in mice by up-regulating integrin beta3 expression in endometrial epithelial cells. Hum Reprod. 2012;27(12):3540-51.

60. Wang J, Rout UK, Bagchi IC, Armant DR. Expression of calcitonin receptors in mouse preimplantation embryos and their function in the regulation of blastocyst differentiation by calcitonin. Development. 1998;125(21):4293-302.

61. Lin XH, Liu ME, Xu HY, Chen XJ, Wang H, Tian S, et al. Leptin down-regulates gamma-ENaC expression: a novel mechanism involved in low endometrial receptivity. Fertil Steril. 2015;103(1):228-35 e3.

62. Lu YC, Yang J, Ding GL, Shi S, Zhang D, Jin L, et al. Small-conductance, calcium-activated potassium channel 3 (SK3) is a modulator of endometrial remodeling during endometrial growth. The Journal of clinical endocrinology and metabolism. 2014;99(10):3800-10.

63. Galgani M, Insabato L, Cali G, Della Gatta AN, Mirra P, Papaccio F, et al. Regulatory T cells, inflammation, and endoplasmic reticulum stress in women with defective endometrial receptivity. Fertil Steril. 2015;103(6):1579-86 e1.

64. Usadi RS, Murray MJ, Bagnell RC, Fritz MA, Kowalik AI, Meyer WR, et al. Temporal and morphologic characteristics of pinopod expression across the secretory phase of the endometrial cycle in normally cycling women with proven fertility. Fertil Steril. 2003;79(4):970-4.

65. Martel D, Monier MN, Roche D, Psychoyos A. Hormonal dependence of pinopode formation at the uterine luminal surface. Hum Reprod. 1991;6(4):597-603.

66. Bentin-Ley U, Pedersen B, Lindenberg S, Larsen JF, Hamberger L, Horn T. Isolation and culture of human endometrial cells in a three-dimensional culture system. Journal of reproduction and fertility. 1994;101(2):327-32.

67. Kabir-Salmani M, Nikzad H, Shiokawa S, Akimoto Y, Iwashita M. Secretory role for human uterodomes (pinopods): secretion of LIF. Molecular human reproduction. 2005;11(8):553-9.

68. Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA. Mucins in the mucosal barrier to infection. Mucosal immunology. 2008;1(3):183-97.

69. Hey NA, Graham RA, Seif MW, Aplin JD. The polymorphic epithelial mucin MUC1 in human endometrium is regulated with maximal expression in the implantation phase. The Journal of clinical endocrinology and metabolism. 1994;78(2):337-42.

70. Gipson IK, Ho SB, Spurr-Michaud SJ, Tisdale AS, Zhan Q, Torlakovic E, et al. Mucin genes expressed by human female reproductive tract epithelia. Biology of reproduction. 1997;56(4):999-1011.

71. Gipson IK, Blalock T, Tisdale A, Spurr-Michaud S, Allcorn S, Stavreus-Evers A, et al. MUC16 is lost from the uterodome (pinopode) surface of the receptive human endometrium: in vitro evidence that MUC16 is a barrier to trophoblast adherence. Biology of reproduction. 2008;78(1):134-42.

72. Pimental RA, Julian J, Gendler SJ, Carson DD. Synthesis and intracellular trafficking of Muc-1 and mucins by polarized mouse uterine epithelial cells. The Journal of biological chemistry. 1996;271(45):28128-37.

73. Yamada N, Kitamoto S, Yokoyama S, Hamada T, Goto M, Tsutsumida H, et al. Epigenetic regulation of mucin genes in human cancers. Clinical epigenetics. 2011;2(2):85-96.

74. Xu B, Sun X, Li L, Wu L, Zhang A, Feng Y. Pinopodes, leukemia inhibitory factor, integrin-beta3, and mucin-1 expression in the peri-implantation endometrium of women with unexplained recurrent pregnancy loss. Fertil Steril. 2012.

75. Tepass U, Truong K, Godt D, Ikura M, Peifer M. Cadherins in embryonic and neural morphogenesis. Nat Rev Mol Cell Biol. 2000;1(2):91-100.

76. Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell. 1996;84(3):345-57.

77. Fujimoto J, Ichigo S, Hori M, Tamaya T. Alteration of E-cadherin, alpha- and beta-catenin mRNA expression in human uterine endometrium during the menstrual cycle. Gynecol Endocrinol. 1996;10(3):187-91.

78. Riethmacher D, Brinkmann V, Birchmeier C. A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. Proc Natl Acad Sci U S A. 1995;92(3):855-9.

79. Li Q, Wang J, Armant DR, Bagchi MK, Bagchi IC. Calcitonin down-regulates E-cadherin expression in rodent uterine epithelium during implantation. The Journal of biological chemistry. 2002;277(48):46447-55.

80. Shirane A, Wada-Hiraike O, Tanikawa M, Seiki T, Hiraike H, Miyamoto Y, et al. Regulation of SIRT1 determines initial step of endometrial receptivity by controlling E-cadherin expression. Biochem Biophys Res Commun. 2012;424(3):604-10.

81. Apparao KB, Murray MJ, Fritz MA, Meyer WR, Chambers AF, Truong PR, et al. Osteopontin and its receptor alphavbeta(3) integrin are coexpressed in the human endometrium during the menstrual cycle but regulated differentially. The Journal of clinical endocrinology and metabolism. 2001;86(10):4991-5000.

82. Wang J, Armant DR. Integrin-mediated adhesion and signaling during blastocyst implantation. Cells Tissues Organs. 2002;172(3):190-201.

83. Illera MJ, Cullinan E, Gui Y, Yuan L, Beyler SA, Lessey BA. Blockade of the alpha(v)beta(3) integrin adversely affects implantation in the mouse. Biology of reproduction. 2000;62(5):1285-90.

84. Illera MJ, Lorenzo PL, Gui YT, Beyler SA, Apparao KB, Lessey BA. A role for alphavbeta3 integrin during implantation in the rabbit model. Biology of reproduction. 2003;68(3):766-71.

85. Chen G, Xin A, Liu Y, Shi C, Chen J, Tang X, et al. Integrins beta1 and beta3 are biomarkers of uterine condition for embryo transfer. J Transl Med. 2016;14(1):303.

86. Kaneko Y, Lecce L, Day ML, Murphy CR. beta(1) and beta(3) integrins disassemble from basal focal adhesions and beta(3) integrin is later localised to the apical plasma membrane

of rat uterine luminal epithelial cells at the time of implantation. Reprod Fertil Dev. 2011;23(3):481-95.

87. Kumar V, Soni UK, Maurya VK, Singh K, Jha RK. Integrin beta8 (ITGB8) activates VAV-RAC1 signaling via FAK in the acquisition of endometrial epithelial cell receptivity for blastocyst implantation. Sci Rep. 2017;7(1):1885.

88. Chung TW, Park MJ, Kim HS, Choi HJ, Ha KT. Integrin alphaVbeta3 and alphaVbeta5 are required for leukemia inhibitory factor-mediated the adhesion of trophoblast cells to the endometrial cells. Biochem Biophys Res Commun. 2016;469(4):936-40.

89. Skrzypczak J, Mikolajczyk M, Szymanowski K. Endometrial receptivity: expression of alpha3beta1, alpha4beta1 and alphaVbeta1 endometrial integrins in women with impaired fertility. Reprod Biol. 2001;1(2):85-94.

90. Germeyer A, Savaris RF, Jauckus J, Lessey B. Endometrial beta3 integrin profile reflects endometrial receptivity defects in women with unexplained recurrent pregnancy loss. Reproductive biology and endocrinology : RB&E. 2014;12:53.

91. Alon R, Feigelson S. From rolling to arrest on blood vessels: leukocyte tap dancing on endothelial integrin ligands and chemokines at sub-second contacts. Semin Immunol. 2002;14(2):93-104.

92. Nejatbakhsh R, Kabir-Salmani M, Dimitriadis E, Hosseini A, Taheripanah R, Sadeghi Y, et al. Subcellular localization of L-selectin ligand in the endometrium implies a novel function for pinopodes in endometrial receptivity. Reproductive biology and endocrinology : RB&E. 2012;10:46.

93. Lai TH, Shih Ie M, Vlahos N, Ho CL, Wallach E, Zhao Y. Differential expression of L-selectin ligand in the endometrium during the menstrual cycle. Fertil Steril. 2005;83 Suppl 1:1297-302.

94. Foulk RA, Zdravkovic T, Genbacev O, Prakobphol A. Expression of L-selectin ligand MECA-79 as a predictive marker of human uterine receptivity. J Assist Reprod Genet. 2007;24(7):316-21.

95. Wang B, Sheng JZ, He RH, Jin F, Huang HF. High expression of L-selectin ligand in secretory endometrium is associated with better endometrial receptivity and facilitates embryo implantation in human being. Am J Reprod Immunol. 2008;60(2):127-34.

96. van de Stolpe A, van der Saag PT. Intercellular adhesion molecule-1. J Mol Med (Berl). 1996;74(1):13-33.

97. Prefumo F, Semino C, Melioli G, Venturini PL. A defective expression of ICAM-1 (CD54) on secretory endometrial cells is associated with endometriosis. Immunol Lett. 2002;80(1):49-53.

98. Wu MH, Yang BC, Lee YC, Wu PL, Hsu CC. The differential expression of intercellular adhesion molecule-1 (ICAM-1) and regulation by interferon-gamma during the pathogenesis of endometriosis. Am J Reprod Immunol. 2004;51(5):373-80.

99. Kuessel L, Wenzl R, Proestling K, Balendran S, Pateisky P, Yotova s, et al. Soluble VCAM-1/soluble ICAM-1 ratio is a promising biomarker for diagnosing endometriosis. Hum Reprod. 2017;32(4):770-9.

100. Comba C, Bastu E, Dural O, Yasa C, Keskin G, Ozsurmeli M, et al. Role of inflammatory mediators in patients with recurrent pregnancy loss. Fertil Steril. 2015;104(6):1467-74 e1.

101. Lee CL, Lam MP, Lam KK, Leung CO, Pang RT, Chu IK, et al. Identification of CD147 (basigin) as a mediator of trophoblast functions. Hum Reprod. 2013;28(11):2920-9.

102. Kuno N, Kadomatsu K, Fan QW, Hagihara M, Senda T, Mizutani S, et al. Female sterility in mice lacking the basigin gene, which encodes a transmembrane glycoprotein belonging to the immunoglobulin superfamily. FEBS Lett. 1998;425(2):191-4.

103. Suzuki N, Zara J, Sato T, Ong E, Bakhiet N, Oshima RG, et al. A cytoplasmic protein, bystin, interacts with trophinin, tastin, and cytokeratin and may be involved in trophinin-mediated cell adhesion between trophoblast and endometrial epithelial cells. Proc Natl Acad Sci U S A. 1998;95(9):5027-32.

104. Tamura N, Sugihara K, Akama TO, Fukuda MN. Trophinin-mediated cell adhesion induces apoptosis of human endometrial epithelial cells through PKC-delta. Cell Cycle. 2011;10(1):135-43.

105. Pan X, Wang X, Wang Z, Wang X, Dou Z, Li Z. Bisphenol a influences blastocyst implantation via regulating integrin beta3 and trophinin expression levels. Int J Clin Exp Med. 2015;8(11):20035-45.

106. Wang HY, Xing FQ, Chen SL. Expression of trophinin in the cycling endometrium and its association with infertility. Di Yi Jun Yi Da Xue Xue Bao. 2002;22(6):539-41.

107. Danielsson KG, Swahn ML, Bygdeman M. The effect of various doses of mifepristone on endometrial leukaemia inhibitory factor expression in the midluteal phase--an immunohistochemical study. Hum Reprod. 1997;12(6):1293-7.

108. Yen CF, Liao SK, Huang SJ, Tabak S, Arcuri F, Lee CL, et al. Decreased Endometrial Expression of Leukemia Inhibitory Factor Receptor Disrupts the STAT3 Signaling in Adenomyosis During the Implantation Window. Reprod Sci. 2017;24(8):1176-86.

109. Tawfeek MA, Eid MA, Hasan AM, Mostafa M, El-Serogy HA. Assessment of leukemia inhibitory factor and glycoprotein 130 expression in endometrium and uterine flushing: a possible diagnostic tool for impaired fertility. BMC Womens Health. 2012;12:10.

110. Simon C, Frances A, Piquette GN, el Danasouri I, Zurawski G, Dang W, et al. Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. Endocrinology. 1994;134(2):521-8.

111. Gonzalez RR, Leavis P. Leptin upregulates beta3-integrin expression and interleukin-1beta, upregulates leptin and leptin receptor expression in human endometrial epithelial cell cultures. Endocrine. 2001;16(1):21-8.

112. Simon C, Gimeno MJ, Mercader A, O'Connor JE, Remohi J, Polan ML, et al. Embryonic regulation of integrins beta 3, alpha 4, and alpha 1 in human endometrial epithelial cells in vitro. The Journal of clinical endocrinology and metabolism. 1997;82(8):2607-16.

113. Lalitkumar PG, Lalitkumar S, Meng CX, Stavreus-Evers A, Bentin-Ley U, et al. Mifepristone, but not levonorgestrel, inhibits human blastocyst attachment to an in vitro endometrial three-dimensional cell culture model. Hum Reprod. 2007;22(11):3031-7.

114. Sherwin JR, Smith SK, Wilson A, Sharkey AM. Soluble gp130 is up-regulated in the implantation window and shows altered secretion in patients with primary unexplained infertility. The Journal of clinical endocrinology and metabolism. 2002;87(8):3953-60.

115. Salamonsen LA, Dimitriadis E, Robb L. Cytokines in implantation. Semin Reprod Med. 2000;18(3):299-310.

116. Jones RL, Stoikos C, Findlay JK, Salamonsen LA. TGF-beta superfamily expression and actions in the endometrium and placenta. Reproduction. 2006;132(2):217-32.

117. Caron PL, Frechette-Frigon G, Shooner C, Leblanc V, Asselin E. Transforming growth factor beta isoforms regulation of Akt activity and XIAP levels in rat endometrium during estrous cycle, in a model of pseudopregnancy and in cultured decidual cells. Reproductive biology and endocrinology : RB&E. 2009;7:80.

118. Gurung S, Werkmeister JA, Gargett CE. Inhibition of Transforming Growth Factor-beta Receptor signaling promotes culture expansion of undifferentiated human Endometrial Mesenchymal Stem/stromal Cells. Sci Rep. 2015;5:15042.

119. Correa LF, Zheng Y, Delaney AA, Khan Z, Shenoy CC, Daftary GS. TGF-beta Induces Endometriotic Progression via a Noncanonical, KLF11-Mediated Mechanism. Endocrinology. 2016;157(9):3332-43.

120. Shen M, Liu X, Zhang H, Guo SW. Transforming growth factor beta1 signaling coincides with epithelial-mesenchymal transition and fibroblast-to-myofibroblast transdifferentiation in the development of adenomyosis in mice. Hum Reprod. 2016;31(2):355-69.

121. Xue X, Chen Q, Zhao G, Zhao JY, Duan Z, Zheng PS. The Overexpression of TGF-beta and CCN2 in Intrauterine Adhesions Involves the NF-kappaB Signaling Pathway. PLoS One. 2015;10(12):e0146159.

122. Oner J, Oner H. Immunolocalization of insulin-like growth factor I (IGF-I) during preimplantation in rat uterus. Growth Horm IGF Res. 2007;17(4):271-8.

123. Herr F, Liang OD, Herrero J, Lang U, Preissner KT, Han VK, et al. Possible angiogenic roles of insulin-like growth factor II and its receptors in uterine vascular adaptation to pregnancy. The Journal of clinical endocrinology and metabolism. 2003;88(10):4811-7.

124. Torry DS, Leavenworth J, Chang M, Maheshwari V, Groesch K, Ball ER, et al. Angiogenesis in implantation. J Assist Reprod Genet. 2007;24(7):303-15.

125. Pollard JW, Hunt JS, Wiktor-Jedrzejczak W, Stanley ER. A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. Dev Biol. 1991;148(1):273-83.

126. Cohen PE, Zhu L, Pollard JW. Absence of colony stimulating factor-1 in osteopetrotic (csfmop/csfmop) mice disrupts estrous cycles and ovulation. Biology of reproduction. 1997;56(1):110-8.

127. Othman R, Omar MH, Shan LP, Shafiee MN, Jamal R, Mokhtar NM. Microarray profiling of secretory-phase endometrium from patients with recurrent miscarriage. Reprod Biol. 2012;12(2):183-99.

128. Jensen JR, Witz CA, Schenken RS, Tekmal RR. A potential role for colony-stimulating factor 1 in the genesis of the early endometriotic lesion. Fertil Steril. 2010;93(1):251-6.

129. Budrys NM, Nair HB, Liu YG, Kirma NB, Binkley PA, Kumar S, et al. Increased expression of macrophage colony-stimulating factor and its receptor in patients with endometriosis. Fertil Steril. 2012;97(5):1129-35 e1.

130. Huang SJ, Zenclussen AC, Chen CP, Basar M, Yang H, Arcuri F, et al. The implication of aberrant GM-CSF expression in decidual cells in the pathogenesis of preeclampsia. Am J Pathol. 2010;177(5):2472-82.

131. Moldenhauer LM, Keenihan SN, Hayball JD, Robertson SA. GM-CSF is an essential regulator of T cell activation competence in uterine dendritic cells during early pregnancy in mice. J Immunol. 2010;185(11):7085-96.

132. Li J, Mo S, Chen Y. The effect of G-CSF on infertile women undergoing IVF treatment: A meta-analysis. Syst Biol Reprod Med. 2017;63(4):239-47.

133. Xie Y, Zhang T, Tian Z, Zhang J, Wang W, Zhang H, et al. Efficacy of intrauterine perfusion of granulocyte colony-stimulating factor (G-CSF) for Infertile women with thin endometrium: A systematic review and meta-analysis. Am J Reprod Immunol. 2017;78(2).

134. Mouhayar Y, Sharara FI. G-CSF and stem cell therapy for the treatment of refractory thin lining in assisted reproductive technology. J Assist Reprod Genet. 2017;34(7):831-7.

135. Lessey BA. The role of the endometrium during embryo implantation. Hum Reprod. 2000;15 Suppl 6:39-50.

136. Stavreus-Evers A, Aghajanova L, Brismar H, Eriksson H, Landgren BM, Hovatta O. Co-existence of heparin-binding epidermal growth factor-like growth factor and pinopodes in human endometrium at the time of implantation. Molecular human reproduction. 2002;8(8):765-9.

137. Fang CX, Nong YQ, Liu FH, Fan L, Chen Y. Heparin-Binding Epidermal Growth Factor-Like Growth Factor Enhances Aquaporin 3 Expression and Function During Mouse Embryo Implantation. Reprod Sci. 2017;24(3):463-70.

138. Aghajanova L, Bjuresten K, Altmae S, Landgren BM, Stavreus-Evers A. HB-EGF but not amphiregulin or their receptors HER1 and HER4 is altered in endometrium of women with unexplained infertility. Reprod Sci. 2008;15(5):484-92.

139. Koji T, Chedid M, Rubin JS, Slayden OD, Csaky KG, Aaronson SA, et al. Progesterone-dependent expression of keratinocyte growth factor mRNA in stromal cells of the primate endometrium: keratinocyte growth factor as a progestomedin. J Cell Biol. 1994;125(2):393-401.

140. Slayden OD, Rubin JS, Lacey DL, Brenner RM. Effects of keratinocyte growth factor in the endometrium of rhesus macaques during the luteal-follicular transition. The Journal of clinical endocrinology and metabolism. 2000;85(1):275-85.

141. Seppala M, Taylor RN, Koistinen H, Koistinen R, Milgrom E. Glycodelin: a major lipocalin protein of the reproductive axis with diverse actions in cell recognition and differentiation. Endocr Rev. 2002;23(4):401-30.

142. Soni C, Karande AA. Glycodelin A suppresses the cytolytic activity of CD8+ T lymphocytes. Mol Immunol. 2010;47(15):2458-66.

143. Mylonas I, Jeschke U, Kunert-Keil C, Shabani N, Dian D, Bauerfeind I, et al. Glycodelin A is expressed differentially in normal human endometrial tissue throughout the menstrual cycle as assessed by immunohistochemistry and in situ hybridization. Fertil Steril. 2006;86(5):1488-97.

144. Focarelli R, Luddi A, De Leo V, Capaldo A, Stendardi A, Pavone V, et al. Dysregulation of GdA Expression in Endometrium of Women With Endometriosis: Implication for Endometrial Receptivity. Reprod Sci. 2017:1933719117718276.

145. Bastu E, Mutlu MF, Yasa C, Dural O, Nehir Aytan A, Celik C, et al. Role of Mucin 1 and Glycodelin A in recurrent implantation failure. Fertil Steril. 2015;103(4):1059-64 e2.

146. Ponnampalam AP, Rogers PA. Cyclic changes and hormonal regulation of annexin IV mRNA and protein in human endometrium. Molecular human reproduction. 2006;12(11):661-9.

147. Jiang YL, Li B, Xing FQ, Wang F, Feng JH. [Study on the relationship between altered expression of annexin A4 and endometrial receptivity during the implantation window in infertile patients with endometriosis]. Zhonghua Fu Chan Ke Za Zhi. 2012;47(5):324-7.

148. Berditchevski F, Odintsova E. Characterization of integrin-tetraspanin adhesion complexes: role of tetraspanins in integrin signaling. J Cell Biol. 1999;146(2):477-92.

149. Miyamoto S, Maruyama A, Okugawa K, Akazawa K, Baba H, Maehara Y, et al. Loss of motility-related protein 1 (MRP1/CD9) and integrin alpha3 expression in endometrial cancers. Cancer. 2001;92(3):542-8.

150. Liu WM, Cao YJ, Yang YJ, Li J, Hu Z, Duan EK. Tetraspanin CD9 regulates invasion during mouse embryo implantation. J Mol Endocrinol. 2006;36(1):121-30.

151. Miyado K, Yamada G, Yamada S, Hasuwa H, Nakamura Y, Ryu F, et al. Requirement of CD9 on the egg plasma membrane for fertilization. Science. 2000;287(5451):321-4.

152. Dominguez F, Simon C, Quinonero A, Ramirez MA, Gonzalez-Munoz E, Burghardt H, et al. Human endometrial CD98 is essential for blastocyst adhesion. PLoS One. 2010;5(10):e13380.

153. Tsumura H, Suzuki N, Saito H, Kawano M, Otake S, Kozuka Y, et al. The targeted disruption of the CD98 gene results in embryonic lethality. Biochem Biophys Res Commun. 2003;308(4):847-51.

154. Matsumoto H, Daikoku T, Wang H, Sato E, Dey SK. Differential expression of ezrin/radixin/moesin (ERM) and ERM-associated adhesion molecules in the blastocyst and uterus suggests their functions during implantation. Biology of reproduction. 2004;70(3):729-36.

155. Lecce L, Kaneko Y, Murphy CR. CD43 is relocated from the basal to the apical plasma membrane of rat uterine epithelial cells by progesterone. Histochem Cell Biol. 2010;133(5):549-55.

156. Kumamoto K, Yang XZ, Hasegawa A, Komori S, Koyama K. CD52 expression is induced in the mouse uterus at the time of embryo implantation. J Reprod Immunol. 2009;82(1):32-9.

157. Wei X, Liu S, Wang X, Yan Q. CD82 expression alters with human endometrial cycles and affects the uterine endometrial receptivity in vitro. Exp Biol Med (Maywood). 2012;237(3):254-62.

158. Gellersen B, Briese J, Oberndorfer M, Redlin K, Samalecos A, Richter DU, et al. Expression of the metastasis suppressor KAI1 in decidual cells at the human maternal-fetal interface: Regulation and functional implications. Am J Pathol. 2007;170(1):126-39.

159. Timologou A, Zafrakas M, Grimbizis G, Miliaras D, Kotronis K, Stamatopoulos P, et al. Immunohistochemical expression pattern of metastasis suppressors KAI1 and KISS1 in endometriosis and normal endometrium. Eur J Obstet Gynecol Reprod Biol. 2016;199:110-5.

160. Liu FS, Dong JT, Chen JT, Hsieh YT, Ho ES, Hung MJ, et al. KAI1 metastasis suppressor protein is down-regulated during the progression of human endometrial cancer. Clin Cancer Res. 2003;9(4):1393-8.

161. von Wolff M, Wang X, Gabius HJ, Strowitzki T. Galectin fingerprinting in human endometrium and decidua during the menstrual cycle and in early gestation. Molecular human reproduction. 2005;11(3):189-94.

162. Xie Y, Li Y, Kong Y. OPN induces FoxM1 expression and localization through ERK 1/2, AKT, and p38 signaling pathway in HEC-1A cells. Int J Mol Sci. 2014;15(12):23345-58.

163. Casals G, Ordi J, Creus M, Fabregues F, Casamitjana R, Quinto L, et al. Osteopontin and alphavbeta3 integrin expression in the endometrium of infertile and fertile women. Reproductive biomedicine online. 2008;16(6):808-16.

164. Xiao Y, Li T, Xia E, Yang X, Sun X, Zhou Y. Expression of integrin beta3 and osteopontin in the eutopic endometrium of adenomyosis during the implantation window. Eur J Obstet Gynecol Reprod Biol. 2013;170(2):419-22.

165. Serafini PC, Silva ID, Smith GD, Motta EL, Rocha AM, Baracat EC. Endometrial claudin-4 and leukemia inhibitory factor are associated with assisted reproduction outcome. Reproductive biology and endocrinology : RB&E. 2009;7:30.

166. Pan XY, Li X, Weng ZP, Wang B. Altered expression of claudin-3 and claudin-4 in ectopic endometrium of women with endometriosis. Fertil Steril. 2009;91(5):1692-9.

167. Pan XY, Li X, Che YC, Li HY, Li X, Zhang Y, et al. Overexpression of claudin-4 may be involved in endometrial tumorigenesis. Oncol Lett. 2013;5(4):1422-6.

168. Salker MS, Christian M, Steel JH, Nautiyal J, Lavery S, Trew G, et al. Deregulation of the serum- and glucocorticoid-inducible kinase SGK1 in the endometrium causes reproductive failure. Nat Med. 2011;17(11):1509-13.

169. Feroze-Zaidi F, Fusi L, Takano M, Higham J, Salker MS, Goto T, et al. Role and regulation of the serum- and glucocorticoid-regulated kinase 1 in fertile and infertile human endometrium. Endocrinology. 2007;148(10):5020-9.

170. Salker MS, Steel JH, Hosseinzadeh Z, Nautiyal J, Webster Z, Singh Y, et al. Activation of SGK1 in Endometrial Epithelial Cells in Response to PI3K/AKT Inhibition Impairs Embryo Implantation. Cell Physiol Biochem. 2016;39(5):2077-87.

171. Salker MS, Hosseinzadeh Z, Alowayed N, Zeng N, Umbach AT, Webster Z, et al. LEFTYA Activates the Epithelial Na+ Channel (ENaC) in Endometrial Cells via Serum and Glucocorticoid Inducible Kinase SGK1. Cell Physiol Biochem. 2016;39(4):1295-306.

172. Inagaki N, Stern C, McBain J, Lopata A, Kornman L, Wilkinson D. Analysis of intra-uterine cytokine concentration and matrix-metalloproteinase activity in women with recurrent failed embryo transfer. Hum Reprod. 2003;18(3):608-15.

173. Skrzypczak J, Wirstlein P, Mikolajczyk M, Ludwikowski G, Zak T. TGF superfamily and MMP2, MMP9, TIMP1 genes expression in the endometrium of women with impaired reproduction. Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society. 2007;45 Suppl 1:S143-8.

174. Jana S, Chatterjee K, Ray AK, DasMahapatra P, Swarnakar S. Regulation of Matrix Metalloproteinase-2 Activity by COX-2-PGE2-pAKT Axis Promotes Angiogenesis in Endometriosis. PLoS One. 2016;11(10):e0163540.

175. Zhang Y, Liu S, Liu Y, Wang Z, Wang X, Yan Q. Overexpression of fucosyltransferase VII (FUT7) promotes embryo adhesion and implantation. Fertil Steril. 2009;91(3):908-14.

176. Zhang YM, Zhang YY, Bulbul A, Shan X, Wang XQ, Yan Q. Baicalin promotes embryo adhesion and implantation by upregulating fucosyltransferase IV (FUT4) via Wnt/beta-catenin signaling pathway. FEBS Lett. 2015;589(11):1225-33.

177. Hager H, Gliemann J, Hamilton-Dutoit S, Ebbesen P, Koppelhus U, Jensen PH. Developmental regulation of tissue transglutaminase during human placentation and expression in neoplastic trophoblast. J Pathol. 1997;181(1):106-10.

178. Kabir-Salmani M, Shiokawa S, Akimoto Y, Sakai K, Sakai K, Iwashita M. Tissue transglutaminase at embryo-maternal interface. The Journal of clinical endocrinology and metabolism. 2005;90(8):4694-702.

179. Zhang D, Lei C, Zhang W. Up-regulated monoamine oxidase in the mouse uterus during the peri-implantation period. Arch Gynecol Obstet. 2011;284(4):861-6.

180. Poulson E, Botros M, Robson JM. Effect of 5-hydroxytryptamine and iproniazid on pregnancy. Science. 1960;131(3407):1101-2.

181. Daftary GS, Taylor HS. Molecular markers of implantation: clinical implications. Curr Opin Obstet Gynecol. 2001;13(3):269-74.

182. Makker A, Goel MM, Nigam D, Bhatia V, Mahdi AA, Das V, et al. Endometrial Expression of Homeobox Genes and Cell Adhesion Molecules in Infertile Women With Intramural Fibroids During Window of Implantation. Reprod Sci. 2017;24(3):435-44.

183. Vasquez YM, Mazur EC, Li X, Kommagani R, Jiang L, Chen R, et al. FOXO1 is required for binding of PR on IRF4, novel transcriptional regulator of endometrial stromal decidualization. Mol Endocrinol. 2015;29(3):421-33.

184. Ujvari D, Jakson I, Babayeva S, Salamon D, Rethi B, Gidlof S, et al. Dysregulation of In Vitro Decidualization of Human Endometrial Stromal Cells by Insulin via Transcriptional Inhibition of Forkhead Box Protein O1. PLoS One. 2017;12(1):e0171004.

185. Kurihara I, Lee DK, Petit FG, Jeong J, Lee K, Lydon JP, et al. COUP-TFII mediates progesterone regulation of uterine implantation by controlling ER activity. PLoS Genet. 2007;3(6):e102.

186. Li Q, Kannan A, DeMayo FJ, Lydon JP, Cooke PS, Yamagishi H, et al. The antiproliferative action of progesterone in uterine epithelium is mediated by Hand2. Science. 2011;331(6019):912-6.

187. Jones A, Teschendorff AE, Li Q, Hayward JD, Kannan A, Mould T, et al. Role of DNA methylation and epigenetic silencing of HAND2 in endometrial cancer development. PLoS Med. 2013;10(11):e1001551.

188. Song H, Lim H, Paria BC, Matsumoto H, Swift LL, Morrow J, et al. Cytosolic phospholipase A2alpha is crucial [correction of A2alpha deficiency is crucial] for 'on-time' embryo implantation that directs subsequent development. Development. 2002;129(12):2879-89.

189. Vilella F, Ramirez L, Berlanga O, Martinez S, Alama P, Meseguer M, et al. PGE2 and PGF2alpha concentrations in human endometrial fluid as biomarkers for embryonic implantation. The Journal of clinical endocrinology and metabolism. 2013;98(10):4123-32.

190. Zhu Q, Jin Y, Wang P, Wang H, Lu B, Wang Z, et al. Expression and function of fatty acid-binding protein 4 in epithelial cell of uterine endometrium. Cell Biol Int. 2015;39(5):540-7.

191. Wang P, Zhu Q, Peng H, Du M, Dong M, Wang H. Fatty Acid-Binding Protein 4 in Endometrial Epithelium Is Involved in Embryonic Implantation. Cell Physiol Biochem. 2017;41(2):501-9.

192. Chen C, Zhao Y, Yu Y, Li R, Qiao J. MiR-125b regulates endometrial receptivity by targeting MMP26 in women undergoing IVF-ET with elevated progesterone on HCG priming day. Sci Rep. 2016;6:25302.

193. Lin SC, Li YH, Wu MH, Chang YF, Lee DK, Tsai SY, et al. Suppression of COUP-TFII by proinflammatory cytokines contributes to the pathogenesis of endometriosis. The Journal of clinical endocrinology and metabolism. 2014;99(3):E427-37.

194. Wang Y, Ma CH, Qiao J. [Differential expression of microRNA in eutopic endometrium tissue during implantation window for patients with endometriosis related infertility]. Zhonghua Fu Chan Ke Za Zhi. 2016;51(6):436-41.

195. Kong C, Sun L, Zhang M, Ding L, Zhang Q, Cheng X, et al. miR-133b Reverses the Hydrosalpinx-induced Impairment of Embryo Attachment Through Down-regulation of SGK1. The Journal of clinical endocrinology and metabolism. 2016;101(4):1478-89.

196. Li Z, Jia J, Gou J, Zhao X, Yi T. MicroRNA-451 plays a role in murine embryo implantation through targeting Ankrd46, as implicated by a microarray-based analysis. Fertil Steril. 2015;103(3):834-4 e4.

197. Rosenbluth EM, Shelton DN, Wells LM, Sparks AE, Van Voorhis BJ. Human embryos secrete microRNAs into culture media--a potential biomarker for implantation. Fertil Steril. 2014;101(5):1493-500.

198. Haouzi D, Mahmoud K, Fourar M, Bendhaou K, Dechaud H, De Vos J, et al. Identification of new biomarkers of human endometrial receptivity in the natural cycle. Hum Reprod. 2009;24(1):198-205.

199. Lessey BA, Castelbaum AJ, Sawin SW, Sun J. Integrins as markers of uterine receptivity in women with primary unexplained infertility. Fertil Steril. 1995;63(3):535-42.

200. Meyer WR, Castelbaum AJ, Somkuti S, Sagoskin AW, Doyle M, Harris JE, et al. Hydrosalpinges adversely affect markers of endometrial receptivity. Hum Reprod. 1997;12(7):1393-8.

201. Dubowy RL, Feinberg RF, Keefe DL, Doncel GF, Williams SC, McSweet JC, et al. Improved endometrial assessment using cyclin E and p27. Fertil Steril. 2003;80(1):146-56.

202. Diaz-Gimeno P, Horcajadas JA, Martinez-Conejero JA, Esteban FJ, Alama P, Pellicer A, et al. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. Fertil Steril. 2011;95(1):50-60, e1-15.

203. Tseng LH, Chen I, Chen MY, Yan H, Wang CN, Lee CL. Genome-based expression profiling as a single standardized microarray platform for the diagnosis of endometrial disorder: an array of 126-gene model. Fertil Steril. 2010;94(1):114-9.

204. J. A. Horcajadas ME, J. Sarasa, J. P. Carrascosa, P. Martinez, J. Aizpurua, S. Munne, editor ER MAP ALLOW THE RELIABLE DETERMINATION OF THE WINDOW OF IMPLANTATION IN INFERTILE WOMEN. ASRM Scientific Congress & Expo; 2016; Salt Palace Convention Center, Salt Lake City, Utah: Fertility and Sterility.

205. J.A. Horcajadas Almansa ME, J. Sarasa, J.P. Carrascosa, P.A. Martínez-Ortiz, J. Aizpurua, S. Munné editor ER map: a new comprehensive and reliable endometrial receptivity test. 32nd Annual Meeting of ESHRE; 2016; Helsinki, Finland: Human Reproduction.

206. Marions L, Hultenby K, Lindell I, Sun X, Stabi B, Gemzell Danielsson K. Emergency contraception with mifepristone and levonorgestrel: mechanism of action. Obstetrics and gynecology. 2002;100(1):65-71.

207. Croxatto HB, Brache V, Pavez M, Cochon L, Forcelledo ML, Alvarez F, et al. Pituitary-ovarian function following the standard levonorgestrel emergency contraceptive dose or a single 0.75-mg dose given on the days preceding ovulation. Contraception. 2004;70(6):442-50.

208. Chabbert-Buffet N, Meduri G, Bouchard P, Spitz IM. Selective progesterone receptor modulators and progesterone antagonists: mechanisms of action and clinical applications. Hum Reprod Update. 2005;11(3):293-307.

209. Spitz IM, Croxatto HB, Salvatierra AM, Heikinheimo O. Response to intermittent RU486 in women. Fertil Steril. 1993;59(5):971-5.

210. Liu JH, Garzo G, Morris S, Stuenkel C, Ulmann A, Yen SS. Disruption of follicular maturation and delay of ovulation after administration of the antiprogesterone RU486. The Journal of clinical endocrinology and metabolism. 1987;65(6):1135-40.

211. Gemzell-Danielsson K, Westlund P, Johannisson E, Swahn ML, Bygdeman M, Seppala M. Effect of low weekly doses of mifepristone on ovarian function and endometrial development. Hum Reprod. 1996;11(2):256-64.

212. Gemzell-Danielsson K, Swahn ML, Svalander P, Bygdeman M. Early luteal phase treatment with mifepristone (RU 486) for fertility regulation. Hum Reprod. 1993;8(6):870-3.

213. Danielsson KG, Swahn ML, Westlund P, Johannisson E, Seppala M, Bygdeman M. Effect of low daily doses of mifepristone on ovarian function and endometrial development. Hum Reprod. 1997;12(1):124-31.

214. Croxatto HB, Salvatierra AM, Croxatto HD, Fuentealba B. Effects of continuous treatment with low dose mifepristone throughout one menstrual cycle. Hum Reprod. 1993;8(2):201-7.

215. Brown A, Cheng L, Lin S, Baird DT. Daily low-dose mifepristone has contraceptive potential by suppressing ovulation and menstruation: a double-blind randomized control trial of 2 and 5 mg per day for 120 days. The Journal of clinical endocrinology and metabolism. 2002;87(1):63-70.

216. Benagiano G, Bastianelli C, Farris M, Brosens I. Selective progesterone receptor modulators: an update. Expert Opin Pharmacother. 2014;15(10):1403-15.

217. Nieman LK, Blocker W, Nansel T, Mahoney S, Reynolds J, Blithe D, et al. Efficacy and tolerability of CDB-2914 treatment for symptomatic uterine fibroids: a randomized, double-blind, placebo-controlled, phase IIb study. Fertil Steril. 2011;95(2):767-72 e1-2.

218. Levens ED, Potlog-Nahari C, Armstrong AY, Wesley R, Premkumar A, Blithe DL, et al. CDB-2914 for uterine leiomyomata treatment: a randomized controlled trial. Obstetrics and gynecology. 2008;111(5):1129-36.

219. Donnez J, Tatarchuk TF, Bouchard P, Puscasiu L, Zakharenko NF, Ivanova T, et al. Ulipristal acetate versus placebo for fibroid treatment before surgery. N Engl J Med. 2012;366(5):409-20.

220. Glasier AF, Cameron ST, Fine PM, Logan SJ, Casale W, Van Horn J, et al. Ulipristal acetate versus levonorgestrel for emergency contraception: a randomised non-inferiority trial and meta-analysis. Lancet. 2010;375(9714):555-62.

221. Glasier A. The rationale for use of Ulipristal Acetate as first line in emergency contraception: biological and clinical evidence. Gynecol Endocrinol. 2014;30(10):688-90.

222. Lalitkumar PG, Berger C, Gemzell-Danielsson K. Emergency contraception. Best Pract Res Clin Endocrinol Metab. 2013;27(1):91-101.

223. Stratton P, Hartog B, Hajizadeh N, Piquion J, Sutherland D, Merino M, et al. A single mid-follicular dose of CDB-2914, a new antiprogestin, inhibits folliculogenesis and endometrial differentiation in normally cycling women. Hum Reprod. 2000;15(5):1092-9.

224. Xie H, Tranguch S, Jia X, Zhang H, Das SK, Dey SK, et al. Inactivation of nuclear Wnt-beta-catenin signaling limits blastocyst competency for implantation. Development. 2008;135(4):717-27.

225. Woodward BJ, Lenton EA, Turner K. Human chorionic gonadotrophin: embryonic secretion is a time-dependent phenomenon. Hum Reprod. 1993;8(9):1463-8.

226. Talwar GP, Singh OM, Gupta SK, Hasnain SE, Pal R, Majumbar SS, et al. The HSD-hCG vaccine prevents pregnancy in women: feasibility study of a reversible safe contraceptive vaccine. Am J Reprod Immunol. 1997;37(2):153-60.

227. Piccinni MP, Scaletti C, Mavilia C, Lazzeri E, Romagnani P, Natali I, et al. Production of IL-4 and leukemia inhibitory factor by T cells of the cumulus oophorus: a favorable microenvironment for pre-implantation embryo development. Eur J Immunol. 2001;31(8):2431-7.

228. Simon C, Valbuena D, Krussel J, Bernal A, Murphy CR, Shaw T, et al. Interleukin-1 receptor antagonist prevents embryonic implantation by a direct effect on the endometrial epithelium. Fertil Steril. 1998;70(5):896-906.

229. Strakova Z, Srisuparp S, Fazleabas AT. Interleukin-1beta induces the expression of insulin-like growth factor binding protein-1 during decidualization in the primate. Endocrinology. 2000;141(12):4664-70.

230. Genbacev OD, Prakobphol A, Foulk RA, Krtolica AR, Ilic D, Singer MS, et al. Trophoblast L-selectin-mediated adhesion at the maternal-fetal interface. Science. 2003;299(5605):405-8.

231. Bagot CN, Troy PJ, Taylor HS. Alteration of maternal Hoxa10 expression by in vivo gene transfection affects implantation. Gene Ther. 2000;7(16):1378-84.

232. Fouladi-Nashta AA, Jones CJ, Nijjar N, Mohamet L, Smith A, Chambers I, et al. Characterization of the uterine phenotype during the peri-implantation period for LIF-null, MF1 strain mice. Dev Biol. 2005;281(1):1-21.

233. Glenister TW. Nidation processes in organ culture. Int J Fertil. 1966;11(4):412-23.

234. Green RM. Report of the Human Embryo Research Panel. Kennedy Inst Ethics J. 1995;5(1):83-4.

235. Hannan NJ, Paiva P, Dimitriadis E, Salamonsen LA. Models for study of human embryo implantation: choice of cell lines? Biology of reproduction. 2010;82(2):235-45.

236. Momeni M, Rahbar MH, Kovanci E. A meta-analysis of the relationship between endometrial thickness and outcome of in vitro fertilization cycles. J Hum Reprod Sci. 2011;4(3):130-7.

237. Liu H, Chen Y, Yan F, Han X, Wu J, Liu X, et al. Ultrasound molecular imaging of vascular endothelial growth factor receptor 2 expression for endometrial receptivity evaluation. Theranostics. 2015;5(2):206-17.

238. Carson DD, Lagow E, Thathiah A, Al-Shami R, Farach-Carson MC, Vernon M, et al. Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. Molecular human reproduction. 2002;8(9):871-9.

239. Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, et al. Global gene profiling in human endometrium during the window of implantation. Endocrinology. 2002;143(6):2119-38.

240. Riesewijk A, Martin J, van Os R, Horcajadas JA, Polman J, Pellicer A, et al. Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. Molecular human reproduction. 2003;9(5):253-64.

241. Haouzi D, Dechaud H, Assou S, De Vos J, Hamamah S. Insights into human endometrial receptivity from transcriptomic and proteomic data. Reproductive biomedicine online. 2012;24(1):23-34.

242. Berlanga O, Bradshaw HB, Vilella-Mitjana F, Garrido-Gomez T, Simon C. How endometrial secretomics can help in predicting implantation. Placenta. 2011;32 Suppl 3:S271-5.

243. Sha AG, Liu JL, Jiang XM, Ren JZ, Ma CH, Lei W, et al. Genome-wide identification of micro-ribonucleic acids associated with human endometrial receptivity in natural and stimulated cycles by deep sequencing. Fertil Steril. 2011;96(1):150-5 e5.

244. Bulletti C, Jasonni VM, Lubicz S, Flamigni C, Gurpide E. Extracorporeal perfusion of the human uterus. Am J Obstet Gynecol. 1986;154(3):683-8.

245. Rinehart CA, Jr., Lyn-Cook BD, Kaufman DG. Gland formation from human endometrial epithelial cells in vitro. In Vitro Cell Dev Biol. 1988;24(10):1037-41.

246. Bentin-Ley U, Horn T, Sjogren A, Sorensen S, Falck Larsen J, Hamberger L. Ultrastructure of human blastocyst-endometrial interactions in vitro. Journal of reproduction and fertility. 2000;120(2):337-50.

247. Bentin-Ley U, Sjogren A, Nilsson L, Hamberger L, Larsen JF, Horn T. Presence of uterine pinopodes at the embryo-endometrial interface during human implantation in vitro. Hum Reprod. 1999;14(2):515-20.

248. Remmele W, Stegner HE. [Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue]. Pathologe. 1987;8(3):138-40.

249. Eliason JF. Pegylated cytokines: potential application in immunotherapy of cancer. BioDrugs. 2001;15(11):705-11.

250. Hilton DJ, Nicola NA, Waring PM, Metcalf D. Clearance and fate of leukemia-inhibitory factor (LIF) after injection into mice. J Cell Physiol. 1991;148(3):430-9.

251. Menkhorst E, Zhang JG, Sims NA, Morgan PO, Soo P, Poulton IJ, et al. Vaginally administered PEGylated LIF antagonist blocked embryo implantation and eliminated non-target effects on bone in mice. PLoS One. 2011;6(5):e19665.

252. Aschenbach LC, Hester KE, McCann NC, Zhang JG, Dimitriadis E, Duffy DM. The LIF receptor antagonist PEGLA is effectively delivered to the uterine endometrium and blocks LIF activity in cynomolgus monkeys. Contraception. 2013;87(6):813-23.

253. White CA, Zhang JG, Salamonsen LA, Baca M, Fairlie WD, Metcalf D, et al. Blocking LIF action in the uterus by using a PEGylated antagonist prevents implantation: a nonhormonal contraceptive strategy. Proc Natl Acad Sci U S A. 2007;104(49):19357-62.

254. Reel JR, Hild-Petito S, Blye RP. Antiovulatory and postcoital antifertility activity of the antiprogestin CDB-2914 when administered as single, multiple, or continuous doses to rats. Contraception. 1998;58(2):129-36.

255. Hild SA, Reel JR, Hoffman LH, Blye RP. CDB-2914: anti-progestational/anti-glucocorticoid profile and post-coital anti-fertility activity in rats and rabbits. Hum Reprod. 2000;15(4):822-9.

256. Meng CX, Andersson KL, Bentin-Ley U, Gemzell-Danielsson K, Lalitkumar PG. Effect of levonorgestrel and mifepristone on endometrial receptivity markers in a three-dimensional human endometrial cell culture model. Fertil Steril. 2009;91(1):256-64.

257. Lalitkumar S, Boggavarapu NR, Menezes J, Dimitriadis E, Zhang JG, Nicola NA, et al. Polyethylene glycated leukemia inhibitory factor antagonist inhibits human blastocyst implantation and triggers apoptosis by down-regulating embryonic AKT. Fertil Steril. 2013;100(4):1160-9.

258. Kim A, Bridgeman MB. Ulipristal Acetate (ella): A Selective Progesterone Receptor Modulator For Emergency Contraception. P T. 2011;36(6):325-31.

259. Stratton P, Levens ED, Hartog B, Piquion J, Wei Q, Merino M, et al. Endometrial effects of a single early luteal dose of the selective progesterone receptor modulator CDB-2914. Fertil Steril. 2010;93(6):2035-41.

260. Rosario GX, Hondo E, Jeong JW, Mutalif R, Ye X, Yee LX, et al. The LIF-mediated molecular signature regulating murine embryo implantation. Biology of reproduction. 2014;91(3):66.

261. Hess AP, Hamilton AE, Talbi S, Dosiou C, Nyegaard M, Nayak N, et al. Decidual stromal cell response to paracrine signals from the trophoblast: amplification of immune and angiogenic modulators. Biology of reproduction. 2007;76(1):102-17.

262. Baird DT, Brown A, Critchley HO, Williams AR, Lin S, Cheng L. Effect of long-term treatment with low-dose mifepristone on the endometrium. Hum Reprod. 2003;18(1):61-8.

263. Marions L, Danielsson KG, Swahn ML, Bygdeman M. Contraceptive efficacy of low doses of mifepristone. Fertil Steril. 1998;70(5):813-6.

264. Wolf JP, Chillik CF, Dubois C, Ulmann A, Baulieu EE, Hodgen GD. Tolerance of perinidatory primate embryos to RU 486 exposure in vitro and in vivo. Contraception. 1990;41(1):85-92.

265. Bhagwat SR, Chandrashekar DS, Kakar R, Davuluri S, Bajpai AK, Nayak S, et al. Endometrial receptivity: a revisit to functional genomics studies on human endometrium and creation of HGEx-ERdb. PLoS One. 2013;8(3):e58419.

266. Ruiz-Alonso M, Blesa D, Simon C. The genomics of the human endometrium. Biochim Biophys Acta. 2012;1822(12):1931-42.

267. Brosens JJ, Salker MS, Teklenburg G, Nautiyal J, Salter S, Lucas ES, et al. Uterine selection of human embryos at implantation. Sci Rep. 2014;4:3894.

268. Buhring HJ, Streble A, Valent P. The basophil-specific ectoenzyme E-NPP3 (CD203c) as a marker for cell activation and allergy diagnosis. Int Arch Allergy Immunol. 2004;133(4):317-29.

269. Korekane H, Park JY, Matsumoto A, Nakajima K, Takamatsu S, Ohtsubo K, et al. Identification of ectonucleotide pyrophosphatase/phosphodiesterase 3 (ENPP3) as a regulator of N-acetylglucosaminyltransferase GnT-IX (GnT-Vb). The Journal of biological chemistry. 2013;288(39):27912-26.

270. Koot YE, van Hooff SR, Boomsma CM, van Leenen D, Groot Koerkamp MJ, Goddijn M, et al. An endometrial gene expression signature accurately predicts recurrent implantation failure after IVF. Sci Rep. 2016;6:19411.